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ASSESSMENT OF PHYSICAL CAPABILITIES

With special reference to the evaluation of maximal voluntary isometric muscle strength and maximal working capacity

An experimental study on civilian and military subject groups

BY

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Introduction

Determination of physical fitness is a valuable adjunct to other examinations in appraisal of healthy as well as diseased individuals By estimation of the physical capacity a rational selection of personnel for various occupations in for example industry and military service may be facilitated In medical rehabilitation also establishment of the physical state is important since it provides the basis for correct diagnosis for therapy evaluation and prognosis

In determining physical fitness several components may be taken into consideration i e strength velocity precision and endurance. Different forms of physical activity will make their specific demands on these qualities Among abilities used in heavy physical work however muscle strength and endurance may be considered to play a dominating role

During the last few decades technical progress has involved a successive decrease of physical activity for large parts of the population The consequent lowering of the training level implies a reduced preparedness for emergency situations on the part of both individuals and the State A reaction against these tendencies has been evoked among military as well as medical authorities The interest in these problems has initiated physiological investigations in large civilian and military groups These investigations performed during the last few years have *inter alia* included determination of physical working capacity muscular strength and various constitutional factors (56 92) In this connection new points of view have been presented regarding the relation between these different qualities in different subject groups and under different conditions As a continuation of these studies a further elucidation of the methods for assessment of physical fitness in military personnel has been proposed

In the present work the purpose has been to study some problems involved in determination of muscular strength and physical working capacity Among questions to be elucidated the following may be mentioned

PART I

General

1. Biological and methodological variability in evaluation of maximal voluntary isometric muscle strength
2. Interrelationship between muscle-strength values recorded in different parts of the body
3. Average muscular and circulatory changes during basic military service
4. Relationship between work load and duration in maximal physical performances
5. Reproducibility of an index of maximal working capacity
6. Circulatory adaptation during maximal physical work
7. Association between expressions for maximal working capacity on the one hand and muscular and circulatory capacity factors on the other

Methods

For characterization of the material as well as for studies of the relationship between different physiological data a number of investigational techniques were used

Clinical examination

Each subject was interviewed according to a standard questionnaire In formation was collected regarding age place of residence civil occupation and participation in sport activities The history of previous illness was taken and current symptoms asked about with special regard to cardio-pulmonary diseases and acute catarrhal infections The general condition of the subject was noted and physical examination of the heart was performed The systolic and diastolic blood pressure was measured with the aid of a mercury manometer on the right arm diastolic pressure being read at disappearance of Korotkow sounds Only if specially indicated were other organ systems included in the examination This health control was performed by colleagues working in the Military Medical Centre

Anthropometric determinations

Evaluation of various constitutional factors was made according to the principles and methods described by Martin Stolz & Stolz and Lindegård (88 91 122) Some minor modifications were however introduced

The body height was measured to the nearest 0 5 cm with the subjects barefooted The individuals stood with the back of the head against the rule As another expression of skeletal length the size of the tibia was determined The measurement of bone length was performed externally with the aid of an anthropometer and the value recorded in millimetres For the tibia the length was measured between the margin bordering on the tibial femoral articular groove and the distal end of the medial malleolus

For evaluation of skeletal sturdiness the femoral condylar breadth was determined with a special instrument graded in 0 1 mm (96) The femoral condylar breadth was here defined as the linear distance between the medial and lateral surfaces of the condyles The subject was examined in the sitting

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position with the knee joints at 90° The calipers were slightly pressed against the condyles and the instrument held perpendicularly to the long axis of the leg

The skeletal measures were taken only on the right hand side, since comparison of right- and left-side values had shown only minor differences For the femoral condyles the left-side dimensions were smaller than the right-side values by about 0 5 % (*diff* 0 05 cm *P****) For tibia length no significant difference was found between the right and the left side

The amount of fat was estimated by measurement of the skinfold thickness according to Keys & Brozek (82) The regions used were (1) on the back immediately caudally to the scapula, (2) at the axillary border of the pectoralis major muscle at the level of the mamillae, (3) on the abdomen in the midclavicular line at the umbilical level

The body weight was measured with the individuals wearing shorts the values being recorded to the nearest 0 1 kg

Laboratory investigations

Roentgenological determination of heart volume

The heart volume was determined according to the method of Larsson & Kjellberg as modified by Kjellberg *et al* (83 85) The individuals were here investigated in the prone position Calculation of the heart volume was made from two roentgenograms taken simultaneously in the lateral and anterioposterior projections No consideration was paid to the phase of the heart contraction or the respiratory cycle In the technique mentioned the X-ray tube is angled 30 degrees caudally As has been shown by Heilborn & Strandell this procedure may induce a systematic error leading to an overestimation of the heart volume especially in individuals with large thoracic dimensions (54) In the present subject groups consisting mostly of young men of ordinary slim build this error may be relatively small In a series of duplicate determinations on conscripts ($n = 13$) the error of a single determination amounted to 44 ml (3 9 % of the mean value)

Determination of total haemoglobin (THb) and blood volume (TBV)

The total amount of haemoglobin was determined by the alveolar carbon monoxide method as described by Sjostrand and coworkers (90 115) As a rule, duplicate determinations were performed at intervals of less than one week As normal values for adult males 11—12 g per kg body weight have been reported by Holmgren *et al* (68) In the present investigation the standard error of a single determination was found to be 3 7 %

The haemoglobin concentration was determined spectrophotometrically after haemolyzation with ammonium solution duplicate samples of finger blood being used

The total blood volume was calculated from the total amount of haemoglobin and the relative haemoglobin concentration The slight error due to the assumption of a constant haemoglobin concentration in the whole blood was disregarded The mean value found for adult males is here 82 ml per kg body weight according to Holmgren *et al* (68)

Determination of lactic acid concentration

A capillary sample was taken from the finger tip after heating with towels soaked in hot water Special care was taken to avoid any admixture of sweat or other contaminating agents to the sample The blood was analysed according to the method of Barker & Summerson with slight modifications (described in ref 67) Here 0 05 ml of blood was pipetted into 4 ml trichloroacetetic acid After centrifugation of the precipitate the lactic acid was oxidized by concentrated sulphuric acid The acetaldehyde formed was determined colorimetrically through the reaction with p hydroxidiphenyl in the presence of cupric ions In the material investigated by Holmgren the error of a single determination amounted to 3 5 % (67)

Evaluation of isometric muscle strength

See page 21

Assessment of physical working capacity

See page 61

Statistical methods

The data were treated according to current statistical methods

Random selection was performed with the aid of random digit tables (131) For calculation of the significance of differences and of correlation coefficients the *t* test was used If the probability of the findings being due to chance was less than 0 1 per cent ($P = < 0.001$***) it was regarded as highly significant if it was less than 1 per cent but equal to or greater than 0 1 per cent ($P = 0.001$—0.01**) it was denoted as significant if it was

less than 5 per cent but equal to or greater than 1 per cent ($P = 0.01$–0.05^*), it was regarded as probably significant For studies of correlation by regression analysis the methods described by, e g Snedecor were employed (120)

Statistical symbols

n = number of observations

$\bar{x}$ = arithmetic mean

s = standard deviation

$s_{\bar{x}}$ = standard error of the mean

V_{coeff} = coefficient of variation

r = correlation coefficient

s_e = deviation of residuals in linear regression

$diff$ = difference between two means

p = probability

Material

Different military as well as civilian subject groups were investigated To get representative values the individuals were if possible chosen at random from larger populations In some instances where this could not be done the subjects were chosen in such a manner as to give at least a rough cross section of the actual population The military personnel was as a rule ordered to undergo the examinations In one group however where the investigation was part of a selection procedure for special service all examinees were volunteers

Military personnel

The numerically predominant group of personnel in the Swedish Defence System consists of civilian subjects performing their compulsory military service The present investigations were confined to subject groups from this category of national servicemen Before the investigation at the Military Medical Centre these men had all passed two health controls i e at registra tion and at induction pathological cases having then been rejected The resulting selectivity accounts for the subsequent paucity of pathological findings

Only in one case did a suspected pathological Ecg motivate exclusion of the subject Slight respiratory infections were relatively common in such cases the investigation could as a rule be postponed until the subject had recovered usually after a week In some instances however more prolonged infections fractures or other injuries disqualified the subject from continued participation in longitudinal studies

The investigations were consequently concerned only with normal healthy persons normality being defined as absence of abnormal subjective symptoms and objective signs in the examination performed

Series A

From a population of about 700 fully fit conscripts assigned to an infantry regiment in Stockholm (I 1) 100 men were drawn The selection was per formed on the basis of random digit tables Of the individuals selected 89 were investigated during the first two weeks after induction in May—June

960 Some of the subjects participated in further examinations during the period of basic military training (nine months) The subject group was used mainly for elaborating and testing the method for muscle strength evaluation Some of the main characteristics of the series are reported below

Series B

From a contingent of about 700 fully fit conscripts called up for military service in the spring of 1961 100 men were selected at random They belonged to the same regiment as the above-mentioned group and in all essential respects they were similar to that material At the first examination shortly after induction, 93 individuals who were available underwent anthropometric classification and physical fitness assessment Thereafter the examinees were the subject of a follow up study throughout the nine months of basic military training The protracted investigation involved a further reduction of the original material Among the reasons for exclusion were the following deferment of military service removal to other regiments accidental illness at the time of one or several examinations or changed classification as regards military fitness group One condition for inclusion in the study was that the subjects should be fully fit for military service and that they should have taken part in the regular training during the whole period of investigation The characteristics of the original series (B) are compared with those of the remaining group (B_1) All data refer to the examination in May—June 1961 (See Table 1)

Series C

Fifty-eight conscripts fully fit for military service were drawn from a coast artillery regiment in the vicinity of Stockholm (KA 1) The selection of this group could not be performed at random and the results may therefore not strictly represent the population mentioned As far as could be judged from anthropometric data however the subjects did not differ appreciably from average conscripts as regards body build comparison with series A and B showing no statistical difference in this respect Nor did the physical working capacity (PWC_{170}) show any great difference from that generally found at the stage of military training in question i e four months after induction The examinations were performed in April 1961 Some of the main characteristics of this subject group appear from Table 1

Series D

Fifty two conscripts from the same coast artillery regiment as the above-mentioned group were ordered to undergo investigation in February 1962

Table 1 Mean data of investigated subject groups

		A Conscripts in tig 1960	B Conscripts in tig in 1961	B Conscripts in tig in 1961	C Conscripts in tig in 1961	D Conscripts in tig in 1962	E Conscripts in tig in 196	F Cl lli n l inte rs	F Cl lli n lunt ers	G Middle distance runners	H Weight lifters
Number		89	93	58	58	2	3	28	9	23	15
Age years	$\bar{x}$	19 5	19 6	19 6	20 3	20 3	20 3	26 2	28 4	2? 2	7 1
	s	0 5	0 5	0 5	1 0	1 0	1 0	5 1	6 1	3 3	5 5
Occupation											
heavy		16 9	18 3	13 8	1? 5	7 7	7 9	3 6	0	8 7	13 3
intermediate		14 6	30 1	37 9	30 1	69	44 7	7 1	22 2	30 4	20 0
easy		22 5	0 4	19 0	2 0	1 ?	21 1	39 3	41 5	17 1	40 7
sedentary		41 0	31 2	29 3	32 1	14 ?	26 3	50 0	33 3	43 5	?0 0
Residency											
Stockholm area		77 8	71 0	0 7	11 ?	70 0	?1 1	100	100	73 9	86 7
other towns		8 9	8 6	10 3	32	?2 0	60 5	—	—	4 3	—
rural		13 3	20 4	11 0	?3 2	8 0	18 4	—	—	21 8	13 3
Sport											
competition		26 7	30 1	27 6	8 9	50 0	19 2	21 4	22 2	100	100
exercise		25 8	21 5	22 4	28 6	28 8	17 1	25 0	22 2	—	—
no sport		47 7	48 4	50 0	62 5	21 2	15 7	53 6	55 6	—	—
Height cm	$\bar{x}$	178 ?	177 8	178 4	179 4	178 9	178 7	179 8	178 8	182 9	17? 2
	s	6 8	6 2	6 4	6 4	6 2	5 7	6 7	8 4	5 2	5 3
Weight kg	$\bar{x}$	68 3	66 5	67 3	69 6	69 3	70 9	73 5	73 4	69 3	78 8
	s	8 6	7 4	7 7	7 0	8 8	7 3	9 9	11 9	5 2	1? 1
Femur condyle breadth mm	$\bar{x}$	9 8	9 8	9 9	9 8	—	—	10 0	10 2	9 9	9 8
	s	0 5	0 4	0 4	0 ?			0 4	0 5	0 3	0 4
Length of tibia cm	$\bar{x}$	40 3	40 2	40 6	40 4	—	—	40 4	40 2	42 3	38 1
	s	2 3	2 2	2 2	2 3			2 6	2 7	2 4	2 4
Skinfold thickness region I mm	$\bar{x}$	8 0	7 8	7 9	7 8		—	9 4	9 4	6 1	9 1
	s	3 0	2 8	2 6	2 0			4 0	5 7	1 2	2 5
PWC$_{170}$ kpm/min	$\bar{x}$	1 064	1 038	1 058	1 140	1 081	—	1 096	1 09?	1 551	1 177
	s	218	191	199	158	19?		167	135	151	211
PWCmax 0 kpm/min	$\bar{x}$	—	—	—	1 497	1 400	1 174	1 513	1 466	—	—
	s				180	213	150	270	213		
Heart volume ml	$\bar{x}$	79?	766	770	783	700	805	—	—	900	869
	s	120	100	101	88	139	109			102	144
Muscle strength kp											
Handgrip right	$\bar{x}$	68 ?	66 5	66 6	68 6	—	—	?0 1	70 1	70 3	80 0
	s	11 0	10 0	8 5	10 3			10 9	12 3	11 3	9 9
Elbow fl "	$\bar{x}$	29 4	28 3	28 ?	30 5	—	—	29 6	30 3	30 0	37 7
	s	4 3	3 0	3 5	4 0			3 2	4 1	3 1	3 8
Knee ext "	$\bar{x}$	53 1	50 0	50 6	49 7	—	—	?0 9	47 3	52 6	71 4
	s	12 3	8 5	8 6	8 6			7 7	6 9	5 6	10 5

Note In certain examinations only part of the subject groups participated The reduced number of individuals will in what follows be noted in each case

Sele t 1 at ran lom from an infantry regim nt (I 1) Non elected grou p from a oast artillery regiment (KA 1) N vy s en participating in an pilt 1 exam inati n f r d marine servi e

about one month after induction No systematic random selection was performed but a cross section of physically fully fit conscripts belonging to the regiment was aimed at Here too however a good correspondence was obtained for anthropometric data *compared with other material selected* at random In this group of subjects studies on the maximal physical working capacity were made See Table 1

Series E

For a study of the reproducibility *of maximal dynamic work at different* loads a group of 36 men from the Navy was used The investigation was performed in February 1962 at a time when the subjects had participated in military training for about one month They had all volunteered for the study and the investigation was part of an aptitude examination for submarine service Consequently their motivation and physical data may not be representative of Navy men in general

Civilian subject groups

Series F

Twenty-eight male individuals, including students colleagues and personnel of the Military Medical Centre volunteered in a series of investigations This subject group was more heterogeneous than the military material as regards age and occupation as well as training status On the other hand they were available for longer and more complicated examinations Evaluation of isometric muscle strength and determination of physical working capacity were performed In some cases however maximal oxygen uptake was also measured *This part of the group was denoted as* F_1

Series G

Two groups of athletes i e middle distance runners and weight lifters were studied The reason for choosing representatives of these branches of sport was *a desire to elucidate the muscular and circulatory effects of* one sided physical training The individuals were all taking part in competition activities They belonged to the elite or the category just below that The weight lifters formed a group of 15 men who had engaged in their specialty for an average of 7 years Eight were referable to the heavyweight two to the middleweight and five to the lightweight category The other group consisted of 23 runners They competed over distances ranging from 800 to 10 000 metres and the average period of competitive activity amounted to 1 5 years The characteristics of Series G appear *in Table 1*

PART II

Evaluation of muscle strength

Earlier investigations

The desire for objective methods in evaluation of muscle strength has generated an abundance of devices and techniques A comprehensive survey of earlier work in this field is given by Hunsicker and Donelly (77) In this chapter only some of the main principles and methods will be mentioned

Muscle force may be measured either isotonically or isometrically Methods for isotonic determination are described by Mosso Hellebrandt and DeLorme *et al* (41 55 98) Here the force is measured during the whole range of movement effected by the muscle group in question The procedure is tiring for the individual being tested however and in fact it is the work performed rather than the strength that is measured Because of these disadvantages most investigators have resorted to some form of isometric testing The force is then measured during isometric or almost isometric contractions in certain well-defined positions

In isometric testing the following measuring techniques have been used (a) manual methods (b) mechanical spring balance methods (c) the cable-tension method (d) the mercurial hydraulic method (e) pneumatic systems and (f) strain gauge methods

Most dynamometers have been constructed for determination of the finger flexion force This is the case with the early mechanical balance dynamometers of Regnier Burq Duchenne Brigham Mathieu Collin Smedley and Duvall *et al* (27 42 77 119)

Pneumatic systems have been used by Hamilton and Geckler whereas Tuttle *et al* have more recently devised a hand dynamometer according to the strain gauge principle (48 51 127) For testing the arm and back muscles a modification of the hand dynamometer of Regnier has been used Other spring balance instruments have been constructed by Sargent Vernon and Tuttle *et al* for the back and leg by Shochrin for the leg and by Haxton and Lindegård *et al* for the plantar flexion (53 89 111 113 126 132)

Arm dynamometers have been devised by Galton using mechanical springs and by Darcus Bonde Pedersen and Ralston *et al* applying the strain gauge principle (22 39 47 107)

For a more extensive testing of muscle strength several different systems are available As early as in 1893 Kellogg described a universal dynamometer where the force of 22 muscle groups could be recorded with the aid of a

Method

Principle

The strength in different muscle groups was measured during almost isometric contractions The person investigated had to make attempted movements in standardized positions The force developed was measured with the aid of special dynamometers

Apparatus

For standardization of positions and for fixation of the subject a special chair was designed The dimensions of the seat and of movable arm and leg supports could be adjusted to the size of the individual Furthermore dynamometers attached to the chair could be placed in positions corresponding to certain anatomical points

The dynamometers consisted of elastic steel rods to which strain gauges were glued For temperature compensation one strain gauge was placed on each side of the rod The dimension of the steel rod was chosen according to the measuring range desired for each dynamometer The effective lever of the rod could easily be varied for the purpose of calibration By means of straps or other connections the force of the contracting muscle group was transmitted to the steel At the resulting deformation the electrical resistance of the strain gauges was changed This change was measured by a central recording instrument consisting of a Wheatstone s bridge coupled to an alternating current amplifier[1] Before every testing phase the appropriate dynamometer was connected to the measuring bridge with the aid of a special unit[2]

The capacitance and resistance of the electrical circuit were furthermore adjusted in order to place the pointer of the scale at the initial zero level The forces measured were read directly in kiloponds according to prior calibration

The separate dynamometers had different designs depending upon the

[1] Measuring bridge KWS 11/5 0—1 500 Hz Hottinger

[2] Measuring unit selector UD 6 Hottinger

Note The instrumental equipment was designed in cooperation with K H Mattson and B Widén Institution of Theory and Design of Electrical Machinery Royal Institute of Technology Stockholm

hydraulic system with mercury (81) Lovett *et al* have devised a method by which instead of the concentric and isometric force the eccentric force of different muscles is measured (93) In this so called ' break method is determined the power with which a person can resist an externally applied force In connection with this technique simple spring balances or more complicated dynamometers according to the modification of Schmier have been used (112) A cable-tension method has been developed by Clarke and coworkers In this the force exerted is transmitted by straps and cables to the tension sensitive unit For fixation and standardization of positions a special table and stand enable the cables to be fixed in the desired angles according to the muscle group to be tested The individuals are investigated in the horizontal position and assistants are used to ensure fixation (31)

The perfecting of strain gauge instruments during the last decade has initiated a wider application of this technique in muscle testing In the experimental equipment of Beasley two alternative strain gauge dynamometers are used a so-called manual force gauge and an axial tensiometer gauge *For testing with the latter a system of cables and mechanical couplings* is used in a way similar to that in the method of Clarke (17) Another apparatus using strain gauges has been described by Asmussen *et al* (4) In this method the strength in different muscle groups is mesaured by a number of dynamometers connected to a central recording instrument As this arrangement was found convenient for the purposes of the present investigation equipment similar to the Danish apparatus was chosen *Certain modifications of the* strain-gauge system as well as the fixation arrangements were however found desirable

demands of the testing situation Some of them could however be used for strength determination of several muscle groups The technical equipment and testing procedure are shown in Figs 1 and 2

Procedure

(1) *General*

The person was instructed to perform maximal attempted movements in appropriate positions striving to avoid jerks and other trick movements After the maximal level of contraction had been reached it had to be held for about two or three seconds allowing the investigator to read the value on the potentiometer cale Every attempted movement was performed three times with intervals of about 15—20 seconds If the highest value was reached the third time additional contractions were requested until the maximum of the series was obtained The highest value was used as an expression of the maximal contractile force Each moment of testing was *preceded* by thorough instruction and demonstration of the attempted movements wanted In what follows the standardized positions fixations and dynamometer types used are described for each movement The principal muscles which are mechanically capable of acting in the test movements used are also listed according to current anatomical *and physiological* literature (5 6J 108)

(2) *Description of the muscle test*

Neck

FORWARD FLEXION OF THE NECK (Flexion)

Dynamometer No 1

The individual was investigated in the standing *position* The *dynamometer* with its leather strap was placed horizontally at a level of about 2 cm above the eyebrows of the individual clasping his forehead

Participating muscles m sternocleidomastoideus m rectus capitis ventralis et lateralis mm scaleni mm longus colli et capitis platysma the hyoid muscles when the mandible is fixed by its elevators

BACKWARD FLEXION OF THE NECK (Extension)

The same *dynamometer* and arrangement were used as for forward flexion The strap was however now placed horizontally *around* the back of the head with the individual facing the *dynamometer*

Participating muscles m trapezius mm rectus capitis dorsalis major et

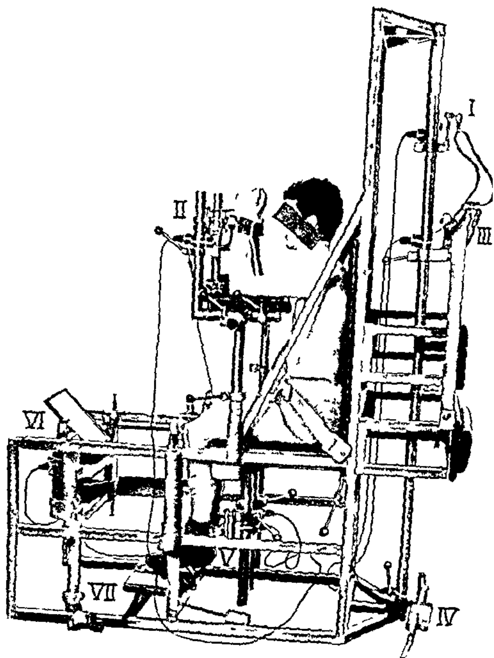


Fig 1 Apparatus for muscle strength determination

Dynamometer	*Application*
No I	neck flexion and extension
II A and B	elbow flexion and extension
III	trunk flexion forwards and backwards
IV	*hip and knee flexion*
V A and B	knee extension
VI	leg extension
VII	foot dorsal and plantar flexion

position between pronation and supination The fastening strap of the *dynamometer* was placed at the level of the styloid process of the radius

Participating muscles m biceps brachii m brachioradialis m brachialis m pronator teres m flexor carpi radialis m palmaris longus m flexor digitorum superficialis m flexor pollicis longus m extensor carpi radialis longus m flexor carpi ulnaris

EXTENSION OF THE ELBOW

Bilateral testing

Dynamometer and positions were the same as for elbow flexion To secure a standard position of 90 flexion in the elbow the upper arm was fixed manually by the assistant At attempted extension the pressure of the wrist was transmitted to the steel rod by a padded plate

Participating muscles m triceps brachii m anconaeus

FINGER FLEXION

Bilateral testing of handgrip

Dynamometer No VIII (see Fig 2 b)

The person was tested in the standing position The handle distance of the *dynamometer* was adjusted to the size of the subject s hand The upper arm was held vertically and the elbow was flexed at 90 The *dynamometer* was supported by the hand not being tested

Participating muscle m flexor digitorum superficialis m flexor digitorum profundus mm flexor pollicis longus et brevis m opponens pollicis m adductor pollicis mm interossei mm lumbricales

Leg

FLEXION OF THE HIP

Bilateral testing

Dynamometer No IX

The subject was investigated in the standing position with the back against a padded support The *dynamometer* with its strap was placed at a level immediately above the patella The subject was instructed to keep the trunk fixed against the support and to flex the hip keeping both knees extended (see Fig 2 f)

Participating muscles m ileopsoas m rectus femoris m tensor fasciae latae m sartorius m gracilis m pectineus mm adductores m obturator ext mm gluteus medius et minimus m quadratus femoris

minor m obliquus capitis, m obliquus atlantis m splenius m longissimus cervicis et capitis m transverso occipitalis mm transversocostales mm interspinales cervicis

Trunk

FORWARD FLEXION OF THE TRUNK

Dynamometer No III

With the individual in the standing position the *dynamometer* was placed with its strap around the chest at a level corresponding to the midpoint of the *sternum* The subject was then instructed to take support with the lower part of the back against a padded plate and to bend forward (see Fig 2 a)

Participating muscles m rectus abdominis mm obliquus abdominis externus et internus, m pyramidalis m rectus femoris, m ileopsoas other hip flexors

BACKWARD FLEXION OF THE TRUNK

The *dynamometer* was the same as for forward flexion but the individual now had to face the measuring unit Support was taken with the pelvis against the plate and the trunk was bent backwards against the *dynamometer* strap

Participating muscles m sacrospinalis, mm interspinales m spinalis m semispinalis mm multifidi

Arm

SHOULDER PULL

Dynamometer No VIII (see Fig 2 c)

The individual was tested in the standing position He was instructed to keep the *dynamometer* in front of the jugular notch and to try to pull the handles apart

Participating muscles m triceps brachii m deltoideus mm teres major et minor mm rhomboides major et minor m trapezius m latissimus dorsi

FLEXION OF THE ELBOW

Bilateral testing

Dynamometers Nos II A and B

The individual was examined sitting in the chair By means of arm supports the upper arms were held horizontally in front of the shoulders at right angles to the trunk The elbow also was held at 90° with the forearm vertically in a

position between pronation and supination The fastening strap of the *dynamometer* was placed at the level of the styloid process of the radius

Participating muscles m biceps brachii m brachioradialis m brachialis m pronator teres m flexor carpi radialis m palmaris longus m flexor digitorum superficialis m flexor pollicis longus m extensor carpi radialis longus m flexor carpi ulnaris

EXTENSION OF THE ELBOW

Bilateral testing

Dynamometer and positions were the same as for elbow flexion To secure a standard position of 90 flexion in the elbow the upper arm was fixed manually by the assistant At attempted extension the pressure of the wrist was transmitted to the steel rod by a padded plate

Participating muscles m triceps brachii m anconaeus

FINGER FLEXION

Bilateral testing of handgrip

Dynamometer No VIII (see Fig 2 b)

The person was tested in the standing position The handle distance of the *dynamometer* was adjusted to the size of the subject s hand The upper arm was held vertically and the elbow was flexed at 90 The *dynamometer* was supported by the hand not being tested

Participating muscles m flexor digitorum superficialis m flexor digitorum profundus mm flexor pollicis longus et brevis m opponens pollicis m adductor pollicis mm interossei mm lumbricales

Leg

FLEXION OF THE HIP

Bilateral testing

Dynamometer No IX

The subject was investigated in the standing position with the back against a padded support The *dynamometer* with its strap was placed at a level immediately above the patella The subject was instructed to keep the trunk fixed against the support and to flex the hip keeping both knees extended (see Fig 2 f)

Participating muscles m iliopsoas m rectus femoris m tensor fasciae latae m sartorius m gracilis m pectineus mm adductores m obturator ext mm gluteus medius et minimus m quadratus femoris

LEG EXTENSION

Combined movement where the composite force of both legs was measured

Dynamometer No VI

The measurement was performed with the subject sitting in the chair with his feet on the plate connected to a strain gauge unit By adjusting the *dynamometer* back and forth it could be fixed in a position such that the angle between the femur and the tibia was about 150° The subject was instructed to take support with the sacrum against the back of the chair and to press the footboard forward (see Fig 2 d)

Participating muscles mm gluteus maximus medius et minimus m *semitendineus m semimembranaceus, m biceps femoris m quadriceps* femoris m adductor magnus m obturator int m piriformis m rectus femoris, m tensor fasciae latae

FLEXION OF THE KNEE

Bilateral testing

Dynamometer No IV

The individual was tested in the standing position The foot of the non tested leg was placed on a platform 5 cm high the tested leg hanging freely The body was fixed by the subject grasping the frame of the chair with both hands and pressing the abdomen and thighs against the supports The *dynamometer* was adjusted to a level where the horizontal strap embraced the Achilles tendon A satisfactory fixation of the trunk and thighs proved important for the obtaining of reproducible results (see Fig 2 e)

Participating muscles m biceps femoris m semitendineus m semimembranaceus m popliteus m gracilis, m sartorius m gastrocnemius m plantaris

EXTENSION OF THE KNEE

Bilateral testing

Dynamometers Nos V A and B

The individual was investigated in the *sitting position* with the legs hanging vertically The straps of the *dynamometers* were placed at the level of the lateral malleolus To secure a standardized position preventing concomitant extension of the hip the pelvis was fixed by a strap coupled to the seat of the chair

Participating muscles m quadriceps femoris m rectus femoris m tensor fasciae latae

PLANTAR FLEXION OF THE FOOT

Bilateral testing

Dynamometer No VII This unit consisted of two metal plates one for each foot mounted on a steel axis connected to the strain gauge The whole *dynamometer* was freely movable in the vertical direction This arrangement was made to prevent participation of the knee and hip extensors

The individual was tested in the sitting position The foot to be measured was fixed to the *dynamometer plate* by a wrist holder whereas the other foot rested on the frame of the chair Thorough instruction combined with demonstration of the attempted movement proved necessary to obtain reliable results The force corresponding to a lever of 30 cm was recorded but this value was then converted to a force corresponding to a certain anatomical level By taking an impression of the foot of the examinee the distance from the axis of the *dynamometer* to the midpoint of the anterior plantar matrix was measured The force recorded was then converted according to this value (see Fig 2 g)

Participating muscles m gastrocnemius m soleus m plantaris m flexor digitorum longus m tibialis posterior mm fibularis longus et brevis m flexor hallucis longus

DORSAL FLEXION OF THE FOOT

Bilateral testing

The same *dynamometer* and position were used as for plantar flexion Instead of the wrist holder which was found to impede the action of the dorsal flexors only a leather strap proximal to the metatarsophalangeal joints was used for fixation and transmission of the force Special attention had to be paid to the exact placing of the foot on the plate to obtain the correct leverage Correction of the force recorded was made according to the foot size in the same manner as for plantar flexion

Participating muscles m tibialis anterior m extensor hallucis longus m extensor digitorum longus m fibularis tertius

Evaluation of method

Instrumental equipment

At the construction of the apparatus a complete calibration against known weights was performed For every strain gauge unit the whole measuring range was checked The values obtained showed a linear relation to successively increasing loads The deviation found was in every case less than $\pm 3\%$ During the whole period of measurement regular calibration controls were

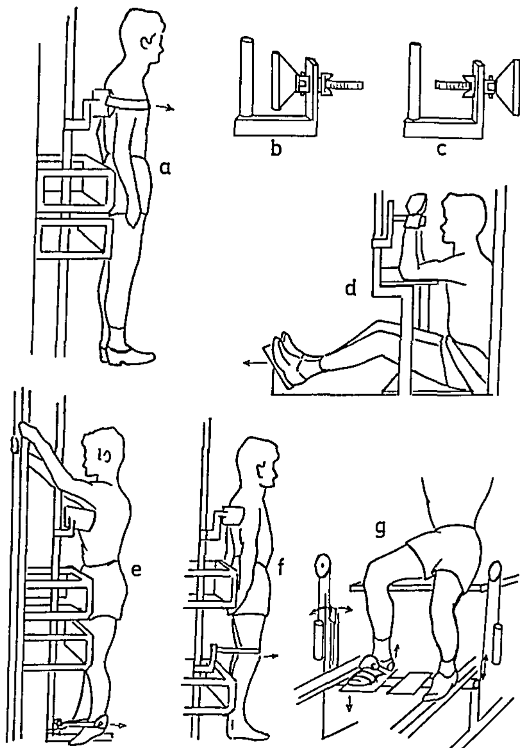


Fig 2 Some attempted movements illustration of apparatus and procedure

a Position used for forward flexion of the trunk
b Dynamometer adapt ı for testing of the finger flexors
c " " " " " shoulder pull
d Position used in leg extension
e " " " knee flexion
f " " hip flexion

performed at intervals of about 10 days Here too the greatest variations noted were less than 3 %

Biological factors

A *Position* An important question in muscle testing is how variations of angles and external levers influence the force measured Some theoretical considerations on this problem have earlier been stated by for instance Gray (49) Practical studies of the elbow flexors have been performed by Wakim *et al* Hunsicker Clarke *et al* and Provins & Salter (33 76 106 130) Similar investigations have been made by Elkins *et al* Clarke *et al* and Provins for the elbow extensors by Elkins *et al* for the shoulder ab ductors by Hettinger for the foot and knee and by Hettinger and Elkins *et al* for the hip flexors (33 45 60) In summary these studies can be said to have shown a great variation in strength of the muscle group when the angle of the corresponding joint was varied The relationship between angle and force was specific for the special muscle group although individual variations were found Alterations of the position of adjacent joints were also shown to influence the strength of a certain muscle group (31 35 110) As expected testing with varying levers established an inverse relationship between force recorded and length of the lever In the investigations report ed there was considerable variation in apparatus as well as testing positions To verify the validity of their results for the present technique a few experi ments were made

Here three individuals were examined with varying angles and levers of some attempted movements The following muscle groups were tested elbow flexion and extension knee extension leg extension and forward and backward flexion of the trunk In principle the results obtained were in close accordance with those of earlier investigators stressing the importance of standardization as regards positions

In designing the testing procedure the following points of view had to be taken into consideration Positions where small changes of the angles will give large alterations of the force are to be avoided The muscular tension and internal leverage of the position chosen must not be disadvantageous to the muscles to be measured The testing positions and fixation arrange- ments must be relatively easy to bring about and must not be uncomfortable for the subject investigated The testing procedure chosen represented a compromise between these different aspects

As regards external leverage the forces were measured at certain anatomical points It seemed more functional to adopt this approach than to use the same distance from the joint in all persons If an approximate proportionality

is presumed to exist between internal and external leverages, the forces measured will here be a rough expression of the physiological cross section of the participating muscles

To study whether the law of leverage was applicable in transformation of forces to torques and vice versa a series of measurements were performed in the elbow and the knee joint The product of force and lever was found to be relatively constant at variation of the lever

B *Sequence of testing* In the present technique a number of muscle groups were examined on the same occasion Ideally the intervals between the muscle contractions ought here to be long enough for complete recovery

A testing procedure where these principles were strictly applied would however, be very time-consuming and tedious It therefore seemed justifiable to use a testing procedure *with constant sequence of testing and with moderately long intervals* To estimate how great are the errors due to fatigue that may be involved in this proceeding, a special investigation was performed Two groups of 12 and 11 individuals respectively were twice examined with one week s interval In one group the same procedure was used both times whereas in the other the sequence was reversed the second time Statistical analysis did not confirm any effect from the change in sequence It ought however to be mentioned that the material investigated was small

C *Effect of repeated tests* In repeated muscle testings the individual may possibly attain a higher value after some time because he has become accustomed to the technique He may thus learn to use the appropriate muscles in a more effective way This learning factor is difficult to isolate since repeated testing may also give some augmentation of strength owing to the training inevitable with the method

In studies where one purpose was to study daily variations the strength was measured three times a week in two individuals for 2½ months (see Fig 3) The strength curves of different muscle groups varied as regards daily fluctuations as well as changes during the testing period In some of them e g forward and backward flexion of the trunk a considerable increase was noted whereas in others such as flexion of the knee there was a moderate decrease Individual differences were also established In plantar flexion of the foot one of the subjects showed a successive increase in strength whereas the other had about constant values Both individuals were conscripts working in the laboratory as assistants Except for the physical exertion involved in calibration of the apparatus none of them had any physical training during the period of investigation

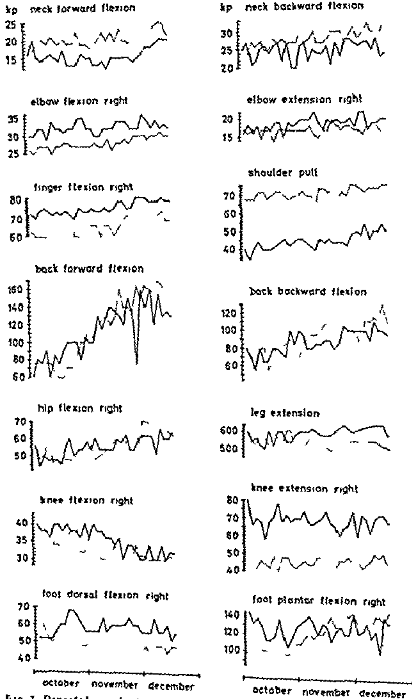


Fig 3 Repeated muscle strength recordings in two individuals studied during 2 ½ months

The subjects in estigated were two 20-year-old laboratory assistants in ordinary training condition All strength values are given in kiloponds

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F *Reproducibility* To establish the reproducibility of the method a series of 44 conscripts was twice investigated with an interval of about 1 ½ hours between each test The results showed considerable variations between the different muscle groups Single movements as for example elbow flexion, where a satisfactory standardization was possible showed a good reproducibility In others however where several joints participated and the movements were complex as for example in flexion of the back the variability was greater The variations found for various muscle groups appear in Table 2

Table 2 Reproducibility at duplicate determinations with about 1½ hours interval

	Attempted movement	$\frac{\bar{x}+\bar{x}}{2}$ kilopond	Diff kilopond	s kilopond	P	Variation coeff %
NECK	forward flexion	18 0	—0 16	1 90	0 2	7.5
	backward flexion	24 7	—0 14	2 01	0 2	5 7
ARM	shoulder pull	48 4	—1 54	4 08		6 0
	elbow flexion right	30 1	—0 47	1 35	*	3 2
	left	29 2	—0 32	1 76	0 2	4 3
	elbow extension right	16 3	—0 44	1 64	0 05	7 1
	left	15 4	—0 39	1 77	0 2	8 1
	finger flexion right	71 6	—0 36	4 48	0 2	4 4
	left	66 8	—0 96	4 77	0 1	5 0
TRUNK	forward flexion	66 9	—0 93	10 1	0 2	10 7
	backward flexion	73 1	+ 1 22	11 8	0 2	11 4
LEG	leg extension	396	—1 59	56 0	0 2	10 0
	hip flexion right	45 9	+ 0 43	4 63	0 2	7 1
	left	44 7	+ 0 71	3 34	0 1	5 3
	knee flexion right	29 9	0 0	2 33	0 2	5.5
	left	29 7	+ 0 48	2 10	0 1	5 0
	knee extension right	51 3	—0 02	3 16	0 2	4 4
	left	48 1	+ 1 00	2 67		3 9
	foot plantar flex r	101 4	—3 76	11 1		7 8
	l	97 4	—1 13	8 9	0 2	6 5
	foot dorsal flex r	42 7	—0 37	3 9	0 2	6 3
	l	41 6	+ 1 46	3 69		6 3

Note Material 44 conscripts 19—20 years old randomly selected from Series A.

Significance of strength recorded with the present method

Maximal voluntary isometric strength is generally accepted as a reliable measure of the ability to perform heavy static or almost static muscular work As other components of muscular capacity static and dynamic endurance may be mentioned Whereas static endurance is rather well correlated with isometric strength (126) the correlation between isometric

In evaluation of the alterations mentioned biological changes during the observation time must be taken into account The increase of values observed may thus partly be due to augmented strength but at least for some movements e g flexion of the back, an enhanced skill cannot be excluded It thus seems probable that the learning error may be of importance mainly for certain muscle groups whereas other movements, such as for example *elbow flexion and finger flexion* may give a good reproducibility even with frequently repeated testings In muscles where alterations were observed *these first became manifest after 6—7 examinations It is therefore reasonable* to assume that the learning error may be reduced if frequent examinations at short intervals are avoided Further support for this view is obtained from duplicate determinations in which no significant alterations between two examinations were observed (See below)

D *Twenty four hours variation* To elucidate the variation of muscular strength during 24 hours three individuals were examined The tests including elbow flexion and knee extension were performed at two hour intervals in the daytime and once during the night The testing was continued with two morning examinations on the following day to reproduce the original values In addition two afternoon values were measured with an interval of some days during the following week The fluctuations observed were small and did not show any uniform relation to any particular time of the day

E *Disposition of the individual* Measurement of the muscle strength involves a volitional process where the attitude of the individual tested will necessarily influence the results Various kinds of external influences may also modify the power (78) Variations due to the disposition of *the individual* may *at least to some degree be reduced in the design of the investigational procedure To stimulate the cooperation of the subjects*—especially the conscripts—it proved to be a good approach to take advantage of their natural competitive instinct In each case the purpose of the investigation was made clear before the examination Afterwards the men obtained their own records giving them the opportunity to compare their own results with those of their fellows With rare exceptions the interest in the own muscle capacity appeared to promote good cooperation To obtain as far as possible uniform investigational conditions *the author performed all investigations* with the aid of an assistant In this way the testing procedure including instruction and *demonstration were as far as* possible standardized

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Attempted movement		$\frac{\bar{x}_1 + \bar{x}_2}{2}$	Diff	kilopond s	P	Variation coeff %
NECK	forward flexion	18 0	− 0 16	1 90	0 2	7 [illegible]
	backward flexion	24 7	− 0 14	2 01	0 2	5 7
ARM	shoulder pull	48 4	−1 54	4 03	*	6 0
	elbow flexion right	30 1	−0 47	1 35		4 2
	» left	29 2	−0 32	1 76	0 2	4 3
	elbow extension right	16 3	−0 44	1 64	0 05	7 1
	» left	15 4	−0 39	1 77	0 2	8 1
	finger flexion right	1 6	−0 6	4 48	0 2	4 4
	» left	[illegible]6 8	−0 96	4 77	0 1	5 0
TRUNK	forward flexion	[illegible]6 9	−0 93	10 1	0 2	10 7
	backward flexion	[illegible]3 1	+ 1 [illegible]2	11 8	0 2	11 4
LEG	leg extension	336	−1 59	56 0	0 2	10 0
	hip flexion right	45 9	+ 0 43	4 63	0 2	7 1
	left	44 [illegible]	+ 0 71	3 34	0 1	5 3
	knee flexion right	29 9	0 0	2 33	0 2	5 5
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	l	97 4	−1 13	8 9	0 2	[illegible] 5
	foot dorsal flex r	4[illegible] 7	−0 37	3 79	0 2	6 3
	l	41 6	+ 1 46	3 [illegible]		6.3

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The most common way of expressing strength is by measurement of anatomically defined forces (31 48 59 119 130) *In* other methods however torques are recorded instead (22 39 60) The system of muscle testing described by Asmussen *et al* partly includes modified procedures designed by earlier investigators Here either forces or *torques are used* *each* for different muscle *groups* At *presentation* of standard values a correction is performed according to individual differences of body height (6) On theoretical grounds it is presumed that the optimal muscle force of an individual might be proportional to the body height in the second power and the muscle torque *to* the height in the third power *The poor correlation* between recorded values *and* height may *be ascribed to the influence* of obscuring factors including the varying training status of the individuals

In the present study no such transformation of values was performed For all muscle groups the strength was recorded in terms of external forces exerted close to the organ used *In this* way the ability to perform heavy *work with the muscle* group could be presumed to be evaluated in a *realistic* way Assuming the internal leverage to be proportional to the external leverage some information may here be obtained regarding the composite physiological cross section of the participating muscles

Besides the *testing* procedure *the instrumental* equipment is another *factor* determining the absolute values to be obtained with a *given method* In a comparative study by Wakim *et al* it was shown that strain gauge instruments will generally give higher readings than mechanical devices such as the cable tensiometer (130) In Sweden perhaps the most commonly used instrument *for estimation of* grip strength is the Collin dynamometer *It therefore seemed* to be of interest to *compare* measurements obtained by using this instrument with those derived from the strain gauge equipment of the author

Before this study was begun both devices were calibrated against known weights whereby *correct values* where obtained at static loading In Table 3 the means dispersions and relative dispersions are given for the two methods

Table 3 *Comparison between strength values recorded with two different dynamometers*

	Collin dyn. kp				Strain-gauge dyn. kp			
	$\bar{x}$	$s_{\bar{x}}$	s	$s\%$	$\bar{x}$	$s_{\bar{x}}$	s	$s\%$
Right handgrip	42 6	0 8	5 2	12 1	67 9	1 3	11 3	16 6
Left	40 9	0 6	5 7	14 0	65 1	1 3	11 8	18 1

Series A – 9 Conscripts investigated shortly after induction

strength and dynamic endurance is reported to be lower (32) As pointed out earlier the method of the author is confined to measuring only isometric maximal volitional strength

The strength of a muscle is a function of the number and the diametres of the fibres in its physiological cross section i e a section passing through all the fibres In a muscle with parallel fibres having the same direction as the tendon the physiological cross section will agree with the anatomical cross section, whereas in for example pinnate muscles this correspondence will be less good (49)

In the majority of movements in the body, several different muscles are usually acting together The strength of single muscles cannot as a rule be evaluated separately Under exceptional conditions as in amputees, where free tendons could be attached to measuring devices the function of isolated muscles has been studied by Ralston *et al* (107) Because of the complexity of muscular mechanics in intact man estimations of the physiological cross section from muscle strength recorded may always involve a series of approximations

Muscle strength may be recorded either as the force exerted at a certain distance from the moving joint or as the torque developed by the acting muscles

In principle general laws of mechanics may be applied to muscle physiology The relation between force and torque is thus defined by the following formula

$$\text{Torque (kilopond} \times \text{cm)} = \text{force (kilopond)} \times \text{lever (cm)}$$

When denoting the strength of a certain muscle group with the use of the torque no correction is made for the body size of the individual tested Thus two subjects of different height may have the same torque in a certain muscle group e g the elbow flexors Because of disparity of levers their ability to lift weights with the muscle group in question will also be different

From a practical point of view it will usually be more convenient to measure the force than the torque This is especially the case with multiarticular movements where the levers are difficult to evaluate cf finger flexion and flexion of the back

For comparison of the results obtained with different methods it is essential to know the principle of investigation the technical equipment the procedure and the standard position used If the muscle strength is expressed different ways it may be impossible to translate values from one method to another

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Series A N = 79 Conscripts investigated shortly after induction

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CHAPTER V

Studies on muscle strength

The present method for evaluation of muscle strength was designed with a view to use in clinical as well as social medicine One of the main aims how ever was to elucidate the possibilities of simple muscle testing in military personnel The first step in this study was to establish the strength pattern in a series of muscle groups of healthy 19/20-year-old men As a second question the interrelationship between values from different parts of the body was investigated The relation to certain anthropometric data was furthermore elucidated To study how environment may modify muscular strength the values were followed in a group of conscripts during the first year of military service

Muscular strength pattern in 19/20 year old conscripts

The method described was tested in a group of conscripts performing their basic military training (Series A) During the investigation modifications had to be made for several testing phases before the technique could be regarded as definite Part of the results could however be used for elucidation of special questions For a longitudinal study of the muscular adaptation during the first year of military service a second group was used (Series B) The selection and characteristics of this group are described above Here the results from the first investigation shortly after the subjects transfer from civilian to military life will be reported

The above mentioned muscle strength values were recorded in a group of healthy 19/20-year-old Swedish males fully fit for military service As mentioned earlier the majority of subjects came from the Stockholm area In civilian life they had mostly light or moderately heavy occupations and 51 % took part in sport either for exercise or as competitors From earlier investigations we know that factors such as sex age body build and occupation are all of importance in determining the degree of muscular strength (6 59 87 128) Although derived from a randomly selected material the values reported may be of restricted representativity

For some of the muscle groups i e handgrip elbow flexors and knee extensors a comparison was performed with a corresponding material (Series A) investigated one year earlier The differences found were small and not

Here, too, the strain gauge method yielded higher absolute values than the mechanical dynamometer In relative terms however, satisfactory agreement was obtained with correlation coefficients of 0 60*** and 0 67*** for the right and left handgrip respectively As pointed out by Wakim *et al* the difference between the absolute values may be ascribed to the fact that mechanical devices have a higher internal friction than strain gauge instruments The same may be said of the divergent dispersions

To sum up, it may be concluded that comparisons between absolute values obtained with two different methods are to be avoided Divergences may be due to the testing procedure and/or the instrumental equipment If differences in these respects are taken into account comparisons may, however, be performed for evaluation of, for example relative changes in two experimental series

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The present method for evaluation of muscle strength was designed with a view to use in clinical as well as social medicine One of the main aims however was to elucidate the possibilities of simple muscle testing in military personnel The first step in this study was to establish the strength pattern in a series of muscle groups of healthy 19/20-year-old men As a second question the interrelationship between values from different parts of the body was investigated The relation to certain anthropometric data was furthermore elucidated To study how environment may modify muscular strength the values were followed in a group of conscripts during the first year of military service

Muscular strength pattern in 19/20 year old conscripts

The method described was tested in a group of conscripts performing their basic military training (Series A) During the investigation modifications had to be made for several testing phases before the technique could be regarded as definite Part of the results could however be used for elucidation of special questions For a longitudinal study of the muscular adaptation during the first year of military service a second group was used (Series B) The selection and characteristics of this group are described above Here the results from the first investigation shortly after the subjects transfer from civilian to military life will be reported

The above mentioned muscle strength values were recorded in a group of healthy 19/20-year-old Swedish males fully fit for military service As mentioned earlier the majority of subjects came from the Stockholm area In civilian life they had mostly light or moderately heavy occupations and 51 % took part in sport either for exercise or as competitors From earlier investigations we know that factors such as sex, age body build and occupation are all of importance in determining the degree of muscular strength (6 59 87 128) Although derived from a randomly selected material the values reported may be of restricted representativity

For some of the muscle groups i e handgrip elbow flexors and knee extensors a comparison was performed with a corresponding material (Series A) investigated one year earlier The differences found were small and not

Table 4 Isometric muscle strength values in 92—93 randomly selected 19/20 year old military conscripts (Series B)

Muscle group	Kilopondsx̄			
	$\bar{x}$	s	$s_{\bar{x}}$	s
Neck flexion	15 4	3 2	0 3	20 8
Neck extension	26 9	5 0	0 5	18 6
Shoulder pull	47 7	10 3	1 1	21 6
Elbow flexion right	28 3	3 6	0 4	12 7
Elbow flexion left	27 7	3 5	0 4	12 6
Elbow extension right	16 3	3 0	0 3	18 4
Elbow extension left	15 2	2 7	0 3	17 8
Finger flexion right	66 5	10 0	1 0	15 0
Finger flexion left	62 3	9 7	1 0	15 6
Back forward flexion	62 1	15 2	1 6	24 5
Back backward flexion	65 4	13 2	1 4	20 2
Leg extension	374	94	10	25 1
Hip flexion right	45 1	8 7	0 9	19 3
Hip flexion left	43 9	8 9	0 9	20 3
Knee flexion right	25 1	4 2	0 4	16 7
Knee flexion left	24 4	4 1	0 4	16 8
Knee extension right	50 0	8 5	0 9	17 0
Knee extension left	47 1	8 2	0 9	17 4
Foot plantar flexion right	103 2	20 6	2 2	20 0
Foot plantar flexion left	97 2	18 2	1 9	18 7
Foot dorsal flexion right	39 2	7 2	0 8	18 4
Foot dorsal flexion left	38 2	7 4	0 8	19 4

statistically significant The agreement established may support the view that the values are fairly representative of isometric muscle strength of Swedish national serviceman at least from this particular recruiting area

As mentioned earlier Asmussen *et al* have described a method using technical equipment similar to that used in the present study Although testing conditions as well as the material investigated are different mean values and standard deviations of comparable strength values are roughly of the same magnitude A detailed comparison is difficult to perform however as in the works of Asmussen *et al* the absolute values of muscle strength are not given but instead relative values corrected for the body height of the individual

The correlation between different muscle groups was studied in the group of conscripts reported above (Series B) The coefficients of correlation between muscle strength values recorded appear in Fig 1

As a rule a highly significant correlation was observed between different muscle groups A good correspondence was especially found between right and left-sided values as well as between synergists and antagonists

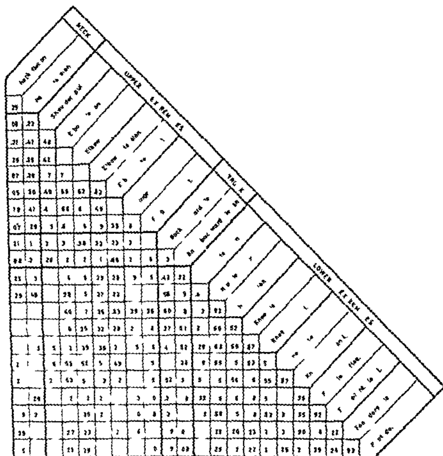


Fig 4 Correlation between isometric muscle strength recorded in different parts of the body

Series B n = 9– 95

The figures denote correlation coefficients (r) In the present study the correlation was found to be statistically highly significant for r = 0 31 statistically significant for r = 0 2 —0 31 and probably significant for r = 0 1—0 27

As expected muscle groups belonging to the same region appeared to be more closely related to one another than to muscles in other parts of the body Also within the regions however disparities were found certain movements such as elbow extension knee flexion and dorsal flexion of the foot showing a somewhat divergent correlation pattern

The correlation between neck strength and the strength of the upper extremities and the trunk was found to be rather low This was also the case

for the power of the trunk compared with that of the upper extremities The correspondence between trunk and leg strength was, on the other hand stronger A relatively good agreement was also found between the values of the upper and lower extremities

Discussion As pointed out by Lindegard, the muscular distribution of strength in a group of individuals will reflect their habitual motional pattern A distinction between different muscles according to their use may here be possible (88)

Theoretically each individual is assumed to have a certain range of muscular capacity, which is genotypically determined The actual phenotypical value may be divided into two fractions viz the basic—or minimal—strength and the strength superimposed thereupon by training The basic strength is supposed to be characteristic for each individual whereas the second part of the actual value, i e the training component, may vary according to the demands of environment

The basic muscle strength is presumably well correlated in different parts of the body *since it is genotypically determined The same assumption may* be valid for the actual muscle strength if the individuals are trained according to their physical preconditions in all muscle groups Selective inactivity or excess training of special groups will however, disturb this correlation

These considerations may aid in elucidating the intraindividual variations of correspondence between different muscle groups in the material investigated As mentioned some muscles, e g neck flexors elbow extensors knee flexors and foot dorsal flexors tended to deviate from the majority of muscle *groups in regard to strength This tendency may at least partly* be explained by an aberrant state of training Other causes such as variability of the measuring method must however also be reckoned with

The conditions mentioned apply to subjects in ordinary physical condition *investigated before military service Different relationships may* be expected to exist in material with a different training status To verify this assumption a number of weight lifters were examined Here remarkably high strength values were found in for example elbow extensors and knee extensors whereas other muscle groups as for example hip flexors seemed to be less trained Compared with the ordinary conscript material the weight lifters showed different pattern of correlation between the different muscle groups This may be demonstrated for the muscles mentioned where a significantly lower correlation was found between hip flexion on the one hand and elbow extension and knee extension in the other as compared with the conscripts

		Athletes	Conscripts
Hip flexion	elbow extension	$r = -0.21$	$r = +0.37$
	knee extension	$r = +0.15$	$r = +0.59$

Prediction of general muscle strength from single muscle group values

The impossibility of determining the total muscularity of an individual will necessarily make all attempts at prediction imperfect. In spite of these limitations there have been devised various methods where different factors are constructed as approximate measures of total muscularity.

Generally a number of muscle groups have been measured and the scores obtained have been combined by simple addition as in the studies of Clarke, Martin *et al.* and Bethe *et al.* (18, 32, 95). The procedure mentioned implies that the various muscle values are weighted, each of them being proportionate to the actual strength of movement. The composite value will, however, greatly depend upon the choice of muscle groups as well as on the testing conditions.

Another method, applied by, for instance, Clarke and Lindegård, is based upon the use of standardized scores (32, 88). Here the strength values recorded are converted to standard deviation units after subtraction of the mean values. The average standardized score—total muscle factor—is used as an expression of the entire strength of the body. The formula of this calculation would be

$$\text{Standardized muscle factor} = \frac{\sum \frac{x - \bar{x}}{s}}{n}$$

x = a single value of muscle strength
$\bar{x}$ = the mean strength value of a single group in the material investigated
s = standard deviation of material
n = number of muscle groups included in the test

In this technique the muscle groups are given equal weight within the resultant muscle factor. One advantage of this procedure is that exclusion of one or several muscle values will induce only a small error of total muscle factor. Sometimes this cannot be avoided, owing to accidental injuries affecting single joints and muscle groups.

The first technique was used by Martin *et al.*, who constructed a so-called total proportional strength index from the values of 22 muscle groups

The strength was determined by their breaking method and in addition the Collin dynamometer technique the latter being used for testing of the handgrip The correlation of the single muscle strength values with the resultant index was studied It was shown that practically all recorded muscle groups correlated well with the total proportional strength according to the definition used For practical purposes an abbreviated test was designed in which only a few of the original testing phases were included It was suggested by the authors that this procedure implied only a slight reduction of accuracy compared to the complete test In the studies reported some important muscle groups such as plantar flexion and hip extension were not included because of technical limitations Similarly the handgrip had to be measured by another method

In principle the procedures mentioned may not add much further information than would studies of the interrelationship between strength values of different muscle groups For comparison with the results of earlier investi

Table 5 Correlation between the strength of single muscle groups and two expressions for estimated total muscle strength (Series B n = 92—93)

Muscle group	Summed muscle factor $\bar{x}$ = 1176 kp		Standardized muscle factor $\bar{x}$ = 0 0 units	
	r	s_e	r	s_e
Neck flexion	0 27**	166	0 33**	0 60
Neck extension	0 52***	147	0 56	0 52
Shoulder pull	0 57***	141	0 59***	0 51
Elbow flexion right	0 61*	133	0 76 *	0 41
Elbow flexion left	0 61***	133	0 75***	0 42
Elbow extension right	0 49***	143	0 67*	0 47
Elbow extension left	0 41 *	155	0 61***	0 50
Handgrip right	0 69***	124	0 68*	0 46
Handgrip left	0 61***	133	0 61***	0 48
Back forward flexion	0 63***	134	0 61***	0 50
Back backward flexion	0 52***	147	0 47***	0 56
Leg extension	0 89***	81	0 61***	0 50
Hip flexion right	0 72* *	120	0 76 **	0 41
Hip flexion left	0 67***	128	0 72 **	0 44
Knee flexion right	0 50***	150	0 61 **	0 49
Knee flexion left	0 58 *	141	0 71***	0 45
Knee extension right	0 70***	124	0 78 **	0 40
Knee extension left	0 69***	124	0 74**	0 43
Foot plantar flex right	0 62***	135	0 69*	0 46
Foot plantar flex left	0 59***	140	0 61 *	0 50
Foot dorsal flex right	0 28**	165	0 40***	0 58
Foot dorsal flex left	0 29**	167	0 38**	0 59

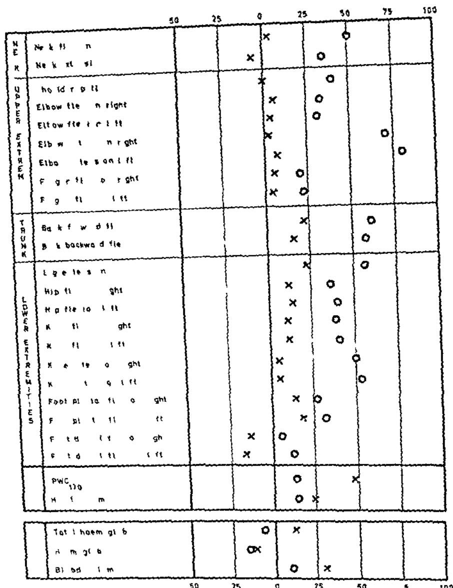


Fig 5 Isometric muscle strength and some circulatory data recorded in two categories of élite athletes as compared with individuals in normal training

Symbol Weight lifters (○) Middle distance runners (×)

Upper section Mean values from weight lifters (Series G $n = 15$) and middle-distance runners (Series [illegible] $n = 23$) expressed as percentage deviation of values from a group of randomly selected conscripts (Series B $n =$ [illegible]—93)

Lower section Mean values from weight lifters ($n =$ [illegible]) and middle distance runners ($n =$ [illegible]) expressed as percentage deviation of normal standard values of the Clinical Physiological Laboratory of Karolinska Sjukhuset As the standard in calculation of relative haemoglobin concentration 15 4 g/100 ml was used For numerical values see Table 6

gators however, the use of similar approximate measures of total muscularity in the present study may be regarded as justifiable From the experimentally obtained strength values two different muscle factors were constructed one using the raw scores and one consisting of average scores converted to standard deviation units The correlation between each of these muscle factors on the one hand and single muscle values on the other is shown in Table 5

As can be seen a good correspondence was generally found between single muscle groups and the above-mentioned muscle factors As pointed out earlier some muscle groups seemed to be less representative of general muscularity than did other muscles In contrast to the findings of Martin *et al*, however the finger flexors showed about the same correlation with the summed muscle factor as did the majority of muscles

The muscle factors mentioned may be used as approximate expressions of the general muscularity of individuals If however, the ability to perform special forms of heavy work is to be estimated a closer definition of its physical demands is desirable For some muscular and circulatory capacity factors this was clearly demonstrated in investigations of two categories of elite athletes See Fig 5 and Table 6

In these studies the effects of selective training were established as muscular and circulatory patterns specific to the appropriate branch of athletic activity As regards muscularity, the factors mentioned may only imperfectly reflect the capacity required in highly specialized occupations of this kind For a better characterization it would be desirable to use some other measure whereby the different muscle values are weighted according to their importance

For special military occupations the physical demands are not known as a whole Certain basic physical capabilities such as a high general muscularity and endurance, may however be assumed to be valuable qualities for elite soldiers A single-muscle strength value which is shown to reflect general muscle strength may here constitute a valuable piece of information in the selection of individuals for physically hard service

For practical reasons muscle groups which are readily accessible for strength determinations and which show a high reproducibility are to be chosen When these factors as well as representativity for estimated general muscularity are taken into consideration such test movements as finger flexion elbow flexion and knee extension may be recommended for abbreviated muscle-strength determinations The above-reported correlation studies furthermore indicated that the leg strength is more closely related to the strength of the back than is the muscle power of the upper extremities A combined testing

of both arm and leg muscle strength will therefore afford a higher accuracy as regards prediction of general strength than will measurements confined to the upper extremity

Table 6 Muscle strength and some circulatory data in two categories of élite athletes

Determination		Weight lifters				Middle-distance runners				Difference	Probability
		n	$\bar{x}$	s	$s_{\bar{x}}$	n	$\bar{x}$	s	$s_{\bar{x}}$		
Neck flexion	kp	15	23.1	2.7	0.7	23	15.9	4.4	0.9	7.2	*
extension		15	36.1	4.0	1.0	23	25.4	1.4	0.9	10.7	
Shoulder pull		15	66.8	10.2	2.6	23	47.6	11.6	2.4	19.2	*
Elbow flexion right		15	37.7	3.8	1.0	23	30.0	3.1	0.7	7.7	***
left		15	36.5	3.3	0.8	23	28.7	3.5	0.7	7.8	*
Elbow extension right		15	27.8	4.1	1.1	23	16.5	2.3	0.5	11.3	
left		15	27.3	3.6	0.9	23	16.2	2.0	0.4	11.1	
Finger flexion right		15	80.0	9.9	2.6	23	70.3	11.3	2.3	9.7	* *
left		15	77.1	8.8	2.3	23	65.0	10.1	[illegible]	12.1	
Back forward flexion		15	100.5	20.6	5.3	23	76.3	20.3	4.2	24.2	*
backward flexion		15	103.9	20.9	5.4	23	75.7	11.9	2.5	28.2	**
Leg extension		15	581	92	21	23	462	93	20	119	*
Hip flexion right		15	61.4	8.9	2.3	23	[illegible]	9.2	1.9	11.1	***
left		15	61.7	9.5	2.5	23	49.9	8.6	1.8	11.8	
Knee flexion right		14	31.8	5.3	1.4	23	27.9	3.5	0.7	6.9	*
left		15	34.0	3.1	0.8	23	27.2	3.5	0.7	6.8	**
Knee extension right		14	74.4	10.5	2.8	23	52.6	5.6	1.2	21.8	**
left		15	72.3	10.4	2.7	22	49.9	5.2	1.1	22.4	*
Foot plantar flexion right		15	131.2	27.9	7.2	21	119.5	18.8	4.1	11.7	0.2–0.1
left		15	128.7	27.7	7.1	21	115.8	18.6	4.1	12.9	0.2–0.1
Foot dorsal flexion right		15	41.8	9.9	2.5	21	34.0	7.4	1.6	7.6	*
left		15	42.8	11.0	2.8	20	32.6	8.1	1.8	10.2	*
PWC_{170} kpm/min		15	1 177	211	55	23	1 531	151	32	374	* *
Heart volume ml		11	869	144	44	22	960	102	[illegible]	91	
Total haemoglobin g		12	790	121	35	22	873	81	17	83	
Haemoglobin conc g/100 ml		12	13.27	0.79	0.23	22	13.43	0.60	0.13	0.16	0.5–0.4
Blood volume l		12	5.97	0.88	0.26	22	6.52	0.68	0.15	0.55	0.1–0.05

Relation between muscle strength and certain anthropometric data

In evaluation of muscle strength the purpose of the investigation must be taken into consideration The best way of expressing strength recorded may vary from situation to situation

For establishment of the ability to perform heavy, mainly static work anatomically defined forces will generally be adequate For follow up studies in connection with physical training or other environmental influences this way of expressing muscle strength will also be satisfactory In other situations however there may be a desire to evaluate muscle strength with regard to the natural preconditions of the individual

The assessment of normal values is a general medical problem and is difficult partly because different definitions may be used for the concept of normality In some works normal muscle strength has been defined as the optimal—mainly genotypically determined—power of an individual when sex body build and age are taken into account In other investigations normal values have been used which are synonymous with the average—phenotypical—strength of individuals having certain biological and social characteristics From this follows the consideration that genotypical and phenotypical strength will show a good correspondence only in subject groups where each individual is trained according to his physical preconditions

In the above mentioned works of Asmussen *et al* attempts were made to establish optimal values for different categories of individuals by correction of recorded strength with regard to body height In the present investigation such conversions have not been attempted Instead it seemed to be of interest to study the actual relation of phenotypical muscle strength to some anthropometric data in the present group of conscripts homogeneous as regards sex and age

As a measure of estimated general strength the standardized muscle factor used earlier was applied In the following table the correlation is set out for some body-build data

Table 7 Relationship between isometric muscle strength (y) and certain anthropometric data (x)

Parameter	Equation of regression line	r	t
Body height cm	$y = 0\ 011\ x + 7\ 98$	0 11	0 3—0 2
Body weight kg	$y = 0\ 017\ x + 6\ 86$	0 56	• •
Tibial length cm	$y = 0\ 019\ x + 9\ 24$	0 08	0 5—0 4
Femoral condylar breadth mm	$y = 0\ 043\ x + 5\ 83$	0 28	

Note Muscle strength is expressed as standardized muscle factor

Discussion As can be seen the correlation between body height and muscle strength was low in the present group of subjects This was the case with the standardized muscle factor as well as with the strength of single muscle groups The other measure of skeletal length recorded i e tibial length also showed a low coefficient of correlation with muscle strength The skeletal breadth on the other hand seemed to be more closely related to muscle strength than was height Concordant results had earlier been obtained by other investigators from studies on similar subject groups (56 88) Of the parameters studied the body weight showed the highest correlation with muscle strength A closer analysis of these conditions is however beyond the scope of this investigation

Muscular and circulatory adaptation in conscripts during military service

During military service people from various occupations and environments are brought together to lead a life in which physical and psychological demands are rather uniform This situation offers exceptional opportunities to study the process of adaptation to altered environmental conditions Apart from their theoretical interest studies of this kind may be of value in the designing of military training programmes

In Sweden there have been performed comprehensive investigations in which one or several physical capacity factors have been followed in conscripts during the year of basic military training (56 87 92) From these studies some general trends of physical adaptation are known As regards muscle strength however our knowledge is still incomplete

In earlier investigations the strength has been recorded in only a few muscle groups all belonging to the upper extremities Many muscles which may be assumed to be of importance for military fitness such as dorsal musculature leg extensors and plantar flexors of the foot have not been included It therefore seemed to be of great interest to apply the present muscle testing method to a longitudinal study of the muscular adaptability in conscripts during the nine months of military service (Series B_1)

Plan of investigation The investigational procedure included clinical examination heart volume determination, complete muscle strength testing anthropometry and determination of the physical working capacity (PWC_{170}) in the order mentioned The first examination was performed during the first two weeks after induction the following ones at intervals of three months the last examination taking place shortly before return to civilian life

In preliminary studies on conscripts during the preceding year there were gained some experiences which seemed to be of value in designing the present investigation Thus it was occasionally found that conscripts hesi tated to show their real capacity because they feared that high records might result in their being given a harder training programme In the actual study the subjects were therefore informed that the results would be used exclusively for scientific purposes and that the individual results would not immediately be imparted to the military authorities The subjects interest and active cooperation was furthermore stimulated by the arrangement that at the end of the investigation everyone was offered the opportunity to become acquainted with his own values

All muscle-strength determinations were performed by the author aided by an assistant At the time of investigation the method had been in use for about a year and initial inaccuracies had been corrected As a rule the examinations were performed between 8 a m and 2 p m

Results The mean values from the repeated muscle-strength measurements of different muscle groups are plotted in Fig 6 For reference the PWC$_{170}$ values the resting pulse and the heart volume in the prone position are also reported

As can be seen, the muscle-strength changes in different parts of the body were found to be rather heterogeneous In some muscle groups the strength showed a tendency to increase during the observation period This was the case with neck flexion shoulder pull, elbow flexion finger flexion and knee flexion In other muscle groups however there was observed an average decline of neck extension back flexion hip flexion and knee extension A third category viz elbow extension and leg extension showed largely un changed values

Generally the mean changes were small, only occasionally exceeding 5 per cent of the initial value sometimes they were not statistically signif icant For some groups where a slight gain was recorded—for example shoulder pull and finger flexion—the highest value was observed shortly before Christmas leave whereas a regression was observed at the last exami nation before departure

The physical working capacity (PWC$_{170}$) which showed a pronounced increase during the first six months, had the same tendency to decline—or stagnate—towards the end of military service The heart-volume alterations approximately followed those of the PWC$_{170}$ whereas the resting pulse showed the opposite direction

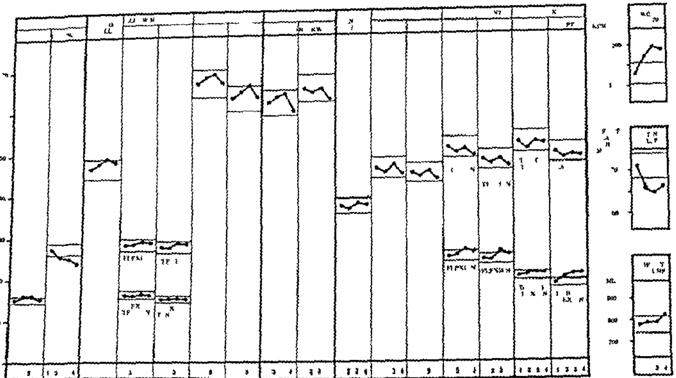


Fig 6 Average changes of isometric muscle strength and of some circulatory factors in 19/20 year old conscripts during basic military service

Series B_1 n = 56—58
The figures at the foot of the graph denote date of examination 1 = May 1961 2 = August 1961 3 = December 1961 4 = March 1962 Horizontal lines above and below experimental curves correspond to the range of ± 5 of initial value All muscle strength values are given in kiloponds For leg extension however the value must be multiplied by 10 and for foot flexions by 2
Numerical values of means standard deviations and standard errors of the mean as well as significance of differences are set out in Tables 8 and 9

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The physical working capacity (PWC_{170}) which showed a pronounced increase during the first six months had the same tendency to decline—or stagnate—towards the end of military service The heart-volume alterations approximately followed those of the PWC_{170} whereas the resting pulse showed the opposite direction

Table 9 Some anthropometric and circulatory factors in conscripts followed during military service (Series B n = 56—58)

P ram ter	M an v l (x)				St nd d ( )				St n l	r of tl n n (s_x)			Stat l significance of differ nc		
Examin tl n N	1	2	3	4	1	2	3	4	1	2	3	4	P = < 0 001	P = 0 001—0 01	P = 0 01—0 1
Body height cm	178 4	178 5	1,8 5	1,8 5	6 1	6 1	6 1	6 4	0 8	0 8	0 8	0 8	—	—	—
Body weight kg	67 3	68 0	68 1	68 8	7 7	7 1	7 0	7 3	1 0	1 0	0 9	0 9	1 1 3—1	?—4	1—3
Femoral condylar breadth cm	9 9	9 9	9 9	9 9	0 39	0 38	0 40	0 39	0 05	0 0	0 0	0 0		—	—
Tibial length cm	40 6	40 0	40 7	40 5			2 2	2 2	0 3	0 3	0 3	0 3	—	—	—
Subcut fat reg I	7 9	7 4	7 3	7 2	2 6	2 0	2 0	2 1	0 31	0 6	0 27	0 25	—	1—? 1—3 1—4	
II	6 3	5 6	5 3	5 3	3 0	2 4	2 1	2 1	0 47	0 31	0 28	0 28	—	1—? 1—3 1—4	—
III	6 8	6 6	6 2	6 0	2 1	2 3	1 9	2 0	0 31	0 30	0 25	0 26	1—4	1—3 ?—4	— 2—3
PWC_{170}	1 058	1 138	1 190	1 171	199	180	173	18.	28	21	23	21	1—2 1—3 1—4	2—3	?—4
Heart volume ml	770	786	786	819	101	9.	93	91	11	13	13	12	1—4 ..—4 3—1	—	1—2 1—3
Pulse resting beats/min	708	6.8	61 7	66 0	11 1	8 6	7 9	9 7	1 5	1 2	1 1	1 3	1—2 1—3	1—4	—
Maximal heart rate beats/min				186 5				8 4				1 1			

Figures denote number of examination

Table 8 Muscular adaptation in conscripts during military service (Series B, n = 56—58)

Muscle group	Mean value ($\bar{x}$) in kiloponds				Stand dev (s)				Stand error of the mean ($s_{\bar{x}}$)				Statist significance of differences		
Examination No	1	2	3	4	1	2	3	4	1	2	3	4	P = < 0 001	P = 0 001 – 0 01	P = 0 01 – 0 1
Neck flexion	15 2	16 6	16 0	15 8	3 3	4 1	3 8	1 0	0 4	0 5	0 5	0 5	1—2 2—4	—	1—3
Neck extension	27 0	25 2	24 4	23 5	4 1	5 1	4 3	4 8	0 5	0 7	0 6	0 6	1—2 3 4 2—4	—	3—4
Shoulder pull	46 6	47 8	49 0	48 1	9 7	9 1	9 7	10 0	1 3	1 2	1 3	1 3	—	—	1—3
Elbow flexion right	28 2	28 5	29 2	28 0	3 5	3 2	3 6	3 5	0 5	0 4	0 5	0 5	—	1—3 2—3	1—4
» left	27 7	27 6	28 6	28 4	3 4	3 5	3 6	3 5	0 5	0 5	0 5	0 5	2—3	1—3 2—4	1—4
Elbow extension right	16 3	16 1	16 4	16 2	2 9	2 8	2 9	3 0	0 4	0 4	0 4	0 4	—	—	—
» left	15 2	15 3	15 4	15 2	2 7	2 7	2 8	2 6	0 4	0 4	0 4	0 3	—	—	—
Finger flexion right	66 6	68 0	68 8	66 7	8 5	7 8	6 9	7 9	1 2	1 1	0 9	1 1	—	3—4	1—3
» » left	62 8	64 4	65 9	63 1	9 3	8 6	8 1	8 2	1 2	1 1	1 1	1 1	1—3 3—4	—	1—2 2—3
Back forward flexion	61 8	62 8	63 8	59 5	16 5	14 1	13 2	14 6	2 2	1 9	1 7	2 0	—	—	3—4
» backward flexion	65 2	64 2	65 1	62 6	11 4	13 0	11 2	12 3	1 5	1 7	1 5	1 6	—	—	1—4
Leg extension	371	363	376	372	89	81	101	96	12	11	14	13	—	—	—
Hip flexion right	45 6	44 8	46 5	44 3	8 2	8 6	8 3	7 9	1 1	1 1	1 1	1 1	3—4	—	2—3
left	44 7	43 9	45 1	43 4	8 9	7 7	8 1	8 8	1 2	1 0	1 1	1 2	—	3—4	—
Knee flexion right	21 9	25 2	26 4	25 9	4 0	3 9	3 8	4 4	0 5	0 5	0 5	0 6	1—3	2—3 1—4	—
» left	21 3	21 2	26 0	25 3	4 1	3 9	4 4	4 1	0 5	0 5	0 6	0 6	1—3 2—3	2—4	1—4
Knee extension right	50 6	49 2	50 1	48 7	8 6	8 1	7 8	8 4	1 1	1 1	1 0	1 1	—	—	1—2 1 3—4
left	47 5	46 7	47 6	46 2	8 2	8 1	8 2	8 0	1 1	1 1	1 1	1 1	—	—	3—4
Foot plantar flexion right	103 5	100 2	103 6	103 0	20 5	16 5	18 0	18 6	2 7	2 2	2 4	2 5	—	—	2—3
left	98 0	91 9	96 5	97 2	19 2	15 9	16 2	17 2	2 6	2 1	2 2	2 3	—	—	—
Foot dorsal flexion right	40 1	40 8	41 7	41 7	7 9	7 8	6 3	6 3	1 1	1 1	0 9	0 9	—	—	1—3 1—4
left	38 6	39 0	40 7	40 7	7 9	8 2	6 3	5 4	1 1	1 1	0 8	0 7	—	1—3	2—3 1—4

Figures denote number of examination

Table 9 Some anthropometric and circulatory factors in conscripts followed during military service (Series B, n = [illegible])

Parameter	Mean value ($\bar{x}$)				Stand dev (s)				Stand err of the mean ($s_{\bar{x}}$)				Statist significance of differences		
Examination No	1	2	3	4	1	2	3	4	1	2	3	4	P = < 0 001	P = 0 001–0 01	P = 0 01–0 1
Body height cm	178 4	1 8 5	1/8 5	178 [illegible]	6 4	[illegible] 1	6 4	6 4	0 8	0 8	0 8	0 8	—	—	—
Body weight kg	67 3	68 0	68 1	68 5	7 7	7 1	7 0	7 3	1 0	1 0	0 9	0 9	1—4 3—4	2—4	1—3
Femoral condylar breadth cm	9 9	9 9	9 9	9 9	0 39	0 38	0 40	0 39	0 05	0 0[illegible]	0 0	0 0[illegible]	—	—	—
Tibial length cm	40 6	40 6	40 7	40 5	2 [illegible]	[illegible]2	2 2	2 2	0 3	0 3	0 3	0 3	—	—	—
Subcut fat reg I	7 9	7 4	7 3	7 2	2 6	2 0	[illegible] 0	2 1	0 31	0 [illegible]6	0 27	0 [illegible]8	—	1—[illegible] 1—3	
II	0 3	[illegible] 6	[illegible] 3	5 3	3 6	2 1	2 1	2 1	0 47	0 31	0 [illegible]8	0 28	—	1—4	—
III	6 8	6 6	6 [illegible]	6 0	2 4	[illegible] 3	1 9	[illegible] 0	0 31	0 30	0 [illegible]	0 26	—	1—2 1—3 1—1	—
PWC_{170}	1 058	1 138	1 190	1 171	199	180	173	185	26	21	23	[illegible]1	1—4	1—3 2—4	2—3
Heart volume ml	770	780	780	819	101	9[illegible]	93	91	14	13	13	12	1—[illegible] 1—3 1—1	2—3	2—1
Pulse resting beat /min	70 8	65 8	61 7	66 0	11 1	8 6	7 9	9 7	1 5	1 2	1 1	1 3	1—1 2—4 3—1	—	1—2 1—3
Maximal heart rate beats/min				186 5				8 4				1 1	1—2 1—3	1—1	—

Figures denote number of examination

Table 8 Muscular adaptation in conscripts during military service (Series B_1 n = 56—58)

Muscle group	Mean value ($\bar{x}$) in kiloponds				Stand dev (s)				Stand error of the mean ($s_{\bar{x}}$)				Statist significance of differences		
Examination No	1	2	3	4	1	2	3	4	1	2	3	4	P = < 0.001	P = 0.001–0.01	P = 0.01–0.1
Neck flexion	15.2	16.6	16.0	15.8	3.3	4.1	3.8	4.0	0.4	0.5	0.5	0.5	1—2 2—4	—	1—3
Neck extension	27.0	25.2	21.4	23.5	4.4	5.1	4.3	4.8	0.5	0.7	0.6	0.6	1—2 3—4 2—4	—	3—4
Shoulder pull	16.6	17.8	49.0	48.1	9.7	9.1	9.7	10.0	1.3	1.2	1.3	1.3	—	—	1—3
Elbow flexion right	28.2	28.5	29.2	28.9	3.5	3.2	3.6	3.5	0.5	0.4	0.5	0.5	—	1—3 2—3	1—4
• • left	27.7	27.6	28.6	28.4	3.4	3.5	3.6	3.5	0.5	0.5	0.5	0.5	2—3	1—3 2—4	1—4
Elbow extension right	16.3	16.1	16.4	16.2	2.9	2.8	2.9	3.0	0.4	0.4	0.4	0.4	—	—	—
• • left	15.2	15.3	15.4	15.2	2.7	2.7	2.8	2.6	0.4	0.4	0.4	0.3	—	—	—
Finger flexion right	66.6	68.0	68.8	66.7	8.5	7.8	6.9	7.9	1.2	1.1	0.9	1.1	—	3—4	1—3
• • left	62.8	61.4	65.9	63.1	9.3	8.6	8.1	8.2	1.2	1.1	1.1	1.1	1—3 3—4	—	1—2 2—3
Back forward flexion	61.8	62.8	63.8	59.5	16.5	14.1	13.2	14.6	2.2	1.9	1.7	2.0	—	—	3—4
• backward flexion	65.2	61.2	65.1	62.6	11.4	13.0	11.2	12.3	1.5	1.7	1.5	1.6	—	—	1—4
Leg extension	371	363	376	372	89	81	101	96	12	11	14	13	—	—	—
Hip flexion right	15.6	11.8	16.5	14.3	8.2	8.6	8.3	7.9	1.1	1.1	1.1	1.1	3—4	—	2—3
• left	11.7	13.9	15.1	13.3	8.9	7.7	8.1	8.8	1.2	1.0	1.1	1.2	—	3—4	—
Knee flexion right	21.9	25.2	26.4	25.9	4.0	3.9	3.8	4.4	0.5	0.5	0.5	0.6	1—3	2—3 1—4	—
left	21.3	21.2	20.0	25.3	4.4	3.9	4.4	4.4	0.5	0.5	0.6	0.6	1—3 2—3	2—4	1—4
Knee extension right	50.6	49.2	50.1	48.7	8.6	8.1	7.8	8.1	1.1	1.1	1.0	1.1	—	—	1—2 1 3—4
• left	47.5	46.7	47.6	46.2	8.2	8.1	8.2	8.0	1.1	1.1	1.1	1.1	—	—	3—4
Foot plantar flexion right	103.5	100.2	103.6	103.0	20.5	16.5	18.0	18.6	2.7	2.2	2.4	2.5	—	—	2—3
• left	98.0	91.9	96.5	97.2	19.2	15.0	16.2	17.2	2.6	2.1	2.2	2.3	—	—	—
Foot dorsal flexion right	40.1	40.8	41.7	41.7	7.9	7.8	6.3	6.3	1.1	1.1	0.9	0.9	—	—	1—3 1—4
left	38.6	39.0	40.7	40.7	7.9	8.2	6.3	5.4	1.1	1.1	0.8	0.7	—	1—3	2—3 1—4

• Figures denote number of examination

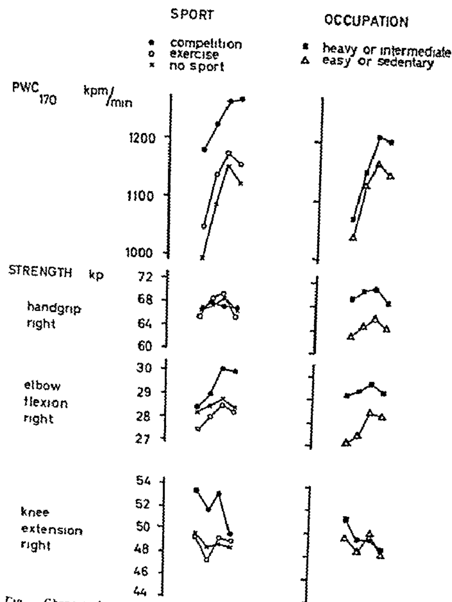


Fig. Changes of isometric muscle strength and PWC_{170} in a group of conscripts divided into subgroups according to occupation and to participation in sports

Series B $n = 58$

Figures at the foot of the graph denote date of examination during basic military service 1 = May 1961, 2 = August 1961, 3 = December 1961 and 4 = March 1962. Number of subjects SPORT competition 16, exercise 13, no sport 29. OCCUPATION heavy or intermediate 30, easy or sedentary 28.

Among the anthropometric data recorded the following may be mentioned Mean body height was unchanged throughout the nine months of observation whereas body weight significantly increased, from 67 3 to 68 8 kg The femoral condylar breadth and the tibial length remained the same The subcutaneous fat was however reduced in all three regions measured the abdominal fat showing the largest reduction

A complete survey of absolute mean values standard deviations, standard errors of mean and significance of differences is given in Tables 8 and 9

Discussion At first sight the changes in muscle strength during the nine months of military service may seem somewhat surprising In particular the occasional decrease in muscle strength in spite of physical training was unexpected To elucidate the mechanism of these changes a closer analysis of the findings was performed with special regard to the composition of the subject group

The material was divided into subgroups according to occupation on the one hand and participation in sport activities on the other Physical working capacity (PWC_{170}) and muscle strength values of five subgroups appear in Fig 7

As regards PWC_{170} the competitive athletes included in the material showed a significantly higher value at the beginning of the observation period than subjects who did not take part in any sport ($P = 0\ 01$—$0\ 001$**) On the other hand the latter group who started on a lower level exhibited the greater relative increase of the two The difference in the changes was however not statistically significant

Comparison between subjects accustomed to heavy work and those previously in light or sedentary occupations showed only a slight non significant difference as regards initial values of PWC_{170} The relative altera tions furthermore seemed to be of the same degree for both categories

Three muscle groups viz finger flexors elbow flexors and knee extensors were studied in the same respect as PWC_{170} For these muscles the physically more active subject groups—the competitive athletes and the individuals used to heavy work—showed higher initial values than did the others The differences were however not statistically significant Although the changes in the subgroups were rather small they may reflect some tendencies to muscular rearrangement As a rule the strength curves of initially less capable subjects and of those with higher initial values had a tendency to converge in the course of military service For knee extension an average decline of strength was observed in the material as a whole This alteration seemed to be due to a selective decrease in initially strong individuals whereas weaker

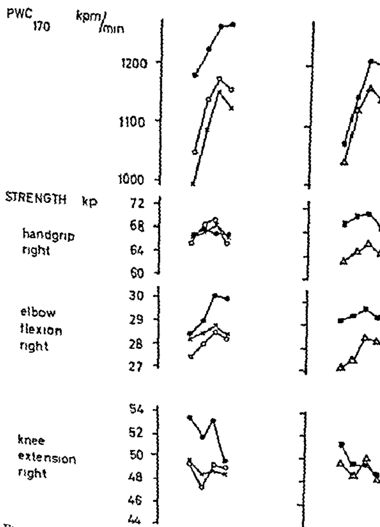


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individuals largely retained their initial muscle strength Nor in this case were the differences between the subgroups statistically significant

Environmental factors such as occupation and participation in sport activities will represent only part of the various civilian influences determining the initial values This may account for the relatively small differences observed between the different subgroups Although not conclusive the tendencies noted may support the view that the initial civilian influences on physical capacity gradually fade during the military service The results reported are in agreement with those of earlier investigators (56 87 92)

As shown earlier, the training level of different muscle groups seems to vary according to their habitual activity The initial variability in combination with the varying muscular demands of military life, may explain the heterogeneity of changes found in different parts of the body

The low degree of changes observed—for most muscles within $\pm 5\%$ of the initial value—would seem to indicate that the general muscular demands of military life are relatively small On the whole they seem to correspond fairly well to those of average civilian life

In order to find out whether any particular period of the training time was more effective than others as regards physical improvement values from the four examinations were compared As mentioned a moderate increase of strength was noted in some muscle groups Here the maximal values were generally reached after about six months, whereas a slight decline was observed towards the end of the training period A similar tendency was found for the physical working capacity and—although in the opposite direction—for the resting pulse

In the present material the average physical working capacity (PWC_{170}) showed a statistically significant increase between induction to military service and departure therefrom A corresponding development was found in 1958—59 by Hellstrom who followed a group of conscripts with a similar civilian background Other conscript groups investigated by Linroth in 1952—53 showed a moderate average decrease of PWC_{170} between two examinations during basic military training As compared with the present finding this may partly be explained by divergent composition of the material studied as regards age civil occupation residence participation in sport etc Partly however it may be due to the fact that the first examination of Linroth was performed 1—2 months after the beginning of military training The initially recorded values in his series were also considerably higher than those observed in the present material

The mean body weight showed a small but statistically significant increase whereas the body height was constant throughout the investigation

Unchanged also were the length of tibia and the distance between the femoral condyles on the right hand side The amount of subcutaneous fat estimated in three regions exhibited a statistically significant decrease

The stationary body height would seem to indicate that skeletal growth had largely ceased in the present material before induction into military service This finding is in close agreement with the results of Linroth s investigations where the mean age was 21 years In Hellstrom s series however a significant increase of body height was recorded during the first three months of military service As this change was observed exclusively during the first phase of the period a small age difference in the two subject groups may account for the disparity Moreover the initial body height of Hellstrom s group was somewhat lower possibly indicating an overrepresentation of individuals whose growth was not entirely completed at the time of induction

The gain of weight followed the increase of physical working capacity whereas the amount of subcutaneous fat showed a contemporaneous reduction The decrease of fatty tissue may probably be attributed to the effects of physical training The gain of weight on the other hand may have different causes It may here be recalled that the increase was relatively small—amounting to 2 2 per cent of the initial weight This alteration may partly be explained by changes of heart size and blood volume connected with circulatory adjustment to physical training Other factors such as for example developmental or seasonal changes of body composition are however also to be reckoned with

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The mean body weight showed a small but statistically significant increase whereas the body height was constant throughout the investigation

PART III

Studies on maximal physical working capacity

The aim of this part of the investigation was to elucidate some problems connected with the evaluation of maximal working capacity. A method earlier described by Grosse-Lordemann & Müller was here applied to investigations of military and civilian subject groups (50).

The average relationship was thus established between work intensity and maximal performance time. The reproducibility of an index of maximal physical working capacity was tested in duplicate determinations on separate days. Exhausting work of varying duration was furthermore studied with regard to circulatory adaptation. The results obtained in subject groups of divergent composition were compared and differences in final heart rate and lactic acid concentration discussed. Finally the present technique was compared with some other methods for assessment of physical capability.

CHAPTER VI

Earlier investigations

Numerous techniques have been devised for evaluation of physical working capacity. The type of test work as well as the technical details varies from method to method. Certain requirements are, however, essential to obtain reproducible results. The work must be fairly simple and familiar to the individuals tested. Furthermore, it must be possible to grade the work intensity objectively. These requirements are largely fulfilled by the work on a bicycle ergometer (61, 71).

The testing method may be either maximal or submaximal. In submaximal tests the work load is generally related to the heart rate (43, 103, 117, 129). These methods have the advantage of objectivity, and they may be applied also to the examination of diseased persons.

Where it is possible to use maximal *performance* tests, they will offer a more realistic way of *estimating the* physical working capacity. In one of the maximal testing methods the greatest *attainable* oxygen uptake—the so called aerobic capacity—is used as an expression of physical working capacity (11, 58, 63, 118, 125). *Although this* measure may afford a reliable value of endurance, the method is hard to apply in large subject groups because of its great technical and personnel requirements.



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Other approaches have been used where the work load is related to the maximal performance time or to that time combined with other factors Among methods using this principle the following may be mentioned

Johnsson & Brouha tested a group of men running on a motor driven treadmill at 7 0 miles per hour on a gradient of 8 % The subjects had to run until exhaustion or for a maximum of 5 minutes From maximal pulse, maximal serum lactate concentration and duration of run a work index was calculated (79 80) Foltz *et al* used a test of exhaustion where double work periods were applied in order to reduce the individual variations (16) Taylor described another method, where the work on a treadmill was gradually increased until the subject became exhausted The maximal time was here used as a so called performance index (121)

Grosse Lordemann & Muller studied the relation between work load and maximal time during which the load could be sustained They used an electrodynamically braked bicycle ergometer of their own design In their experimental series maximal tests with varying loads were performed with 5 *individuals (50 99—102 104) The probable formula reflecting the rela*tionship between work intensity and maximal performance time was established The influence of different variables such as pedalling velocity height of saddle and length of pedals was determined Investigations of the mechanical efficiency were furthermore performed for exhausting work of varying duration In accordance with the formula of the load time curve a value of maximal working capacity could be calculated for each subject investigated Similar investigations were carried out by Bink who studied the relationship between effort and endurance in 5 individuals (20)

From the world records for running various distances the relation between mean velocity and performance time was studied by Hill, Henry Lietzke and Aperia *et al* (1 57 63 86) The data were plotted in a coordinate system and the resultant curve analysed A curve similar to that of Grosse Lordemann & Muller was obtained On the basis of these findings a technique for evaluation of maximal working capacity was suggested using as a measure of physical fitness the work intensity sustainable during a maximum of 6 minutes

In connection with studies on physical performance and perceived exertion a test for short period work on the bicycle ergometer was recently described by Borg (23) Maximal work was here recorded in performances lasting for about 15 seconds using a gradually increasing work load It was suggested that the level attained reflected mainly *muscular capacity* and motivation

Methods

A Submaximal evaluation of physical working capacity (PWC_{170})

As a matter of routine practically all the subjects admitted to the laboratory were investigated according to the method developed at Karolinska Sjukhuset (117 129) The working capacity is expressed as the work intensity which the individual can maintain at a heart rate of 170 beats per minute in circulatory steady state The latter concept was defined as the condition where the pulse rate increased 10 beats or less between the 2nd and 6th minutes at the highest load When the subject did not fulfil this criterion the working capacity was instead related to a pulse level of 160 beats per minute

The working machine used was an electrodynamically braked bicycle ergometer (71) The five ergometers in use were regularly calibrated twice annually In no instance did the variation exceed 3 per cent For the electrocardiographic recordings a direct recording apparatus was used (Mingograph 42 Elema Jarnh Stockholm)

Procedure Prior to the work test an Ecg at rest as well as an orthostatic test was performed The following leads were used standard leads I II and III and precordial leads CR_1 or CH_1 CR_2 or CH_2 CR_4 or CH_4 CR_5 or CH_5 and CR_7 or CH_7 (The head leads were taken during work) For the orthostatic test the heart rate and Ecg reaction were studied after 8 minutes of passive standing The work test was initiated at a load of 300 kpm/min Every 6 minutes the load was increased until the pulse rate reached a level of 170 beats per minute To study the effects of maximal work the test was in some subject groups continued until exhaustion Pulse values were followed at Ecg every two minutes An Ecg was also recorded immediately after the work period and following 4 minutes of rest Using the approximately linear relationship between pulse rate and work load the value of PWC_{170} was obtained by extrapolation or intrapolation

B Maximal oxygen uptake ($V_{O_2 max}$)

In each individual the measurement was preceded by repeated work tests where the maximal performance times were determined for different loads

Other approaches have been used where the work load is related to the maximal performance time or to that time combined with other factors Among methods using this principle the following may be mentioned

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load on the abscissa and the maximal performance time on the ordinate The resultant curve was shown to have a characteristic shape in agreement with the findings of earlier investigators By intra or extrapolation according to the standard form of the curve the relation between work load and maximal performance time could be estimated for different time values As a measure of one type of physical working capacity the work load which could be sustained for a maximum of 6 minutes was chosen Figures representing other kinds of physical working capacity can however be obtained if other points of the experimental curve are used

In connection with these experiments the subjects were trained to breathe into an air collecting system At determination of maximal oxygen uptake the examinees were tested on two or more loads of different severity The load was kept constant during each work period, and the subject had to work until exhaustion

Timed air collections were carried out through a rubber mouthpiece low resistance valve and a short length of tubing leading to a Douglas bag Before collection the tubing system was flushed with exhaled air for ½—1 minute

Sampling was performed repeatedly during each work period and immediately before the end of work two bags were taken in rapid succession From each bag two samples were drawn for duplicate determinations During experiments with a high work load the collection periods varied between 30 and 15 seconds For easier loads the exhaled air was collected during 30—70 seconds

The gas volume was measured at constant suction through a dry gasometer (Nordgas) and collected into recipients The analysis for oxygen and carbon dioxide was performed with the Haldane apparatus From the volume and composition of inhaled and exhaled air the rate of oxygen consumption was obtained for each period correcting for volume change which is apparent from different nitrogen concentrations

C Evaluation of maximal physical working capacity (PWC$_{max}$)

For studies of maximal working capacity the above mentioned technique suggested by Grosse-Lordemann & Muller was utilized Some modifications and additions were however introduced

The work was performed on an electrically braked bicycle ergometer, using a pedal rate of 60 revolutions per minute (71) The effect here remains approximately constant even if the pedalling rate is varied between 50 and 70 r p m

In contrast to the submaximal method described, where the load was increased stepwise during the work period the examinee was here tested on constant loads The examination included several such performances on separate days and with varying loads Each time the work was continued until exhaustion The time of maximal performance was recorded with the aid of a stop-watch The number of work periods was determined by the aim of the special part of the investigation

The values obtained were plotted in a coordinate system with the work

load on the abscissa and the maximal performance time on the ordinate The resultant curve was shown to have a characteristic shape in agreement with the findings of earlier investigators By intra or extrapolation according to the standard form of the curve the relation between work load and maximal performance time could be estimated for different time values As a measure of one type of physical working capacity the work load which could be sustained for a maximum of 6 minutes was chosen Figures representing other kinds of physical working capacity can however be obtained if other points of the experimental curve are used

Dependence of maximal performance time on work intensity

For elucidation of the relation between work intensity and maximal performance time a group of 28 civilian volunteers was investigated (Series F). Each subject here had to participate in 2—5 or occasionally more maximal work tests on separate days. The whole series of performances extended over a period of 2—4 weeks. The intervals amounted to 1—14 days.

The repeated work periods necessary for the study may be suspected to involve some degree of training. To minimize the effects of accidental physical improvement, *the sequence of work intensities was varied irregularly from* individual to individual. The number of performances included in the calculations was furthermore limited to five.

Results. The shape of the curves obtained indicated a linear relationship between logarithmic values of work load and maximal performance time according to the formula suggested by Grosse-Lordemann & Müller (50):

$$\log T = p \cdot \log N + q + z$$

T = time of maximal performance in minutes
N = work load in kilopondmetres per minute
p and q = constants z = disturbing factors

The regression equation was computed for each individual. A high correlation was established between the logarithms of work load and maximal performance time ($r = 0.88$—0.99, $P < 0.001$***). The results seemed to verify that the above-mentioned formula will afford a satisfactory approximation within the time limits studied, viz. between about 1 and 18 minutes. Single values corresponded to a maximal performance time of under 1 minute or more than 18 minutes. In these ranges the experimental data were too scanty to permit definite conclusions regarding the relationship mentioned.

In Table 10 are given the individual results, including values for the constants p and q as well as angle of inclination and estimated physical working capacity during 6 minutes ($PWC_{max\,6}$).

Table 10 Data obtained from maximal performances in 28 civilian volunteers

Subject	Number of work periods	constant p	constant q	angle alpha	$PWC_{max\,6}$ kpm/min
1	4	−3.9[illegible]	+13.42	75.8	1,580
2	3	−4.03	+13.16	76.1	1,180
3	[illegible]	−3.85	+12.89	75.5	1,4[illegible]0
4	[illegible]	5.64	+18.6[illegible]	79.9	1,480
[illegible]	5	−4.96	+16.64	[illegible]8.6	1,[illegible]90
6	4	−3.31	+11.50	73.2	1,690
7	4	−4.43	+15.11	77.3	1,[illegible]00
8	5	4.[illegible]2	+14.00	[illegible]6.7	1,360
9	2	4.[illegible]7	+15.[illegible]6	77.7	1,[illegible]30
10	3	−4.[illegible]0	+15.77	[illegible]8.0	1,5[illegible]0
11	2	−4.08	+14.0[illegible]	76.2	1,[illegible]60
12	3	−6.39	+[illegible]1.24	81.1	1,590
13	3	−4.37	+14.3[illegible]	77.1	1,260
14	2	−11.63	+38.05	85.1	1,[illegible]10
15	[illegible]	4.[illegible]2	+16.11	[illegible]8.0	1,770
16	3	[illegible].56	+18.82	79.8	1,770
17	3	−6.[illegible]	+19.69	80.9	1,060
18	2	−5.00	+15.81	78.7	1,010
19	5	−[illegible].24	+14.13	[illegible]6.7	1,390
20	4	−5.72	+18.96	80.1	1,510
21	5	4.[illegible]8	+15.42	77.7	1,[illegible]0
22	3	−8.73	+29.04	83.[illegible]	1,730
23	5	4.40	+14.99	77.2	1,700
[illegible]	5	−5.3[illegible]	+17.88	[illegible]9.4	1,5[illegible]0
25	[illegible]	−[illegible].19	+1[illegible].00	79.1	1,340
[illegible]	4	−4.77	+16.2[illegible]	8[illegible]	1,780
[illegible]	4	−3.[illegible]5	+11.70	74.3	1,190
[illegible]8	5	−4.6[illegible]	+1[illegible].44	77.8	1,190

The present results were furthermore compared with the findings of Grosse Lordemann & Müller. In Fig. 8 the curves from their five examinees are approximately reproduced together with those of the present study.

As shown above the inclination of experimental double logarithmic curves reveals variations from individual to individual. The inclination was defined by the smallest angle between the regression line and the abscissa (alpha). For each subject this angle was calculated from the tangent of the angle. For the entire group the mean value as well as the standard deviation of the angle were computed.

Besides the above mentioned group of civilian volunteers a series of 52 ordinary conscripts (series D) was investigated in a similar way. In this investigation each individual had to work on *two different* work loads during the same day with an interval of about *4 hours*. On a following day the procedure was reversed. Only data from the first *work period* of the day were used for the present study. The inclination was here computed only from two experimental points.



Fig 8 Double logarithmic graph illustrating the relation between work load and maximal performance time

The broken lines represent results from investigations on five subjects reported by Grosse Lordemann & Müller (100) The continuous lines give the regressions computed from maximal work tests in a group of civilian volunteers (Series I $n = 28$)

In Series I the mean angle was $\bar{x} = 78° 2 \pm 0° 5$ $s = 2° 5$ and in Series D it was $\bar{x} = 78° 6 \pm 1° 1$ $s = 8° 0$ The difference was not statistically significant ($P = 0 8$—$0 7$)

Discussion The relation between work intensity and maximal performance time was shown to imply that the performance time increases in a predictable way as the work load is put lower In the present study the formula suggested by Grosse Lordemann & Müller was confirmed in a larger group of subjects The experiments were however confined to exhausting work with a duration of between about 1 and 18 minutes

The maximal physical working capacity may be defined as the maximal work intensity for a certain type of work which can be sustained during a certain period of time The relationship found between work intensity and maximal performance time indicates that small changes in the maximal physical working capacity will correspond to comparatively large changes in the maximal performance time provided the work intensity is kept constant

As mentioned the inclination of the double logarithmic curve varied from subject to subject The interindividual variation was considerably larger in the conscript group D than in the civilian group F This can at least partly be explained by the inaccuracies involved in calculation of the angle from only two experimental points The small and statistically insignificant difference of inclination between two series furthermore indicated that the interindividual variability may on the whole be relatively moderate

From their investigations Grosse Lordemann & Muller suggested that the variation of inclination of the load time curve may be due to disparities in the relation between aerobic and anaerobic capacities In what follows the importance of these two types of energy supply will be elucidated

As compared with the results of earlier investigators the values of the present group were generally on a higher level The inclination of curves however seemed to vary within about the same limits When comparing the characteristics of the two subject groups it was found that the German subjects were considerably shorter and lighter than the Swedish ones The material of Grosse Lordemann & Muller consisted mainly of laboratory personnel of both sexes whereas the Swedish group included only males usually above average in regard to physical working capacity This may account for the differences observed

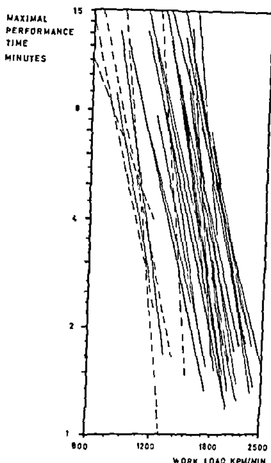


Fig 8 Double logarithmic graph illustrating the relation between work load and maximal performance time

The broken lines represent results from investigations on five subjects reported by Grosse Lordemann & Müller (100) The continuous lines give the regressions computed from maximal work tests in a group of civilian volunteers (Series F $n = 28$)

In Series F the mean angle was $\bar{x} = 78° 2 \pm 0° 5$ $s = 2° 5$ and in Series D it was $\bar{x} = 78° 6 \pm 1° 1$ $s = 8° 0$ The difference was not statistically significant ($P = 0 8—0 7$)

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CHAPTER IX

Reproducibility of estimated maximal working capacity ($PWC_{max\,6}$)

The reproducibility was investigated in a group of conscripts undergoing aptitude tests for submarine service (Series E). Each subject had to participate in duplicate maximal work tests on separate days. The work load, which was the same on both occasions, was chosen to give a maximal performance time of about 6 minutes.

For each experimental value the $PWC_{max\,6}$ *was estimated according* to the following formula

$$\log X = \frac{\log T - \log 6}{tg\ alpha} + \log N$$

X = estimated work load corresponding to maximal performance time of 6 minutes ($PWC_{max\,6}$)

N = work load used

T = *maximal performance time*

alpha = angle between the double logarithmic line and the abscissa

In the above mentioned procedure the $PWC_{max\,6}$-value was estimated from one experimental point according to the empirically established average relationship between work load and maximal performance time. The values were thereby made comparable.

This transformation will involve some degree of approximation, owing to individual *variations of the line. In Fig. 9 some information is given regarding the magnitude of* this error. Here values representing $PWC_{max\,6}$ were calculated for Series F using two *different procedures.* One figure was computed from the regression equation of 2—3 load time values, whereas the other was estimated from only one point according to the mean inclination of the double logarithmic line. The latter, more approximate value was expressed as a percentage of the first, put in relation to the maximal performance time.

The variability was computed for performances of varying duration, *i.e. between 1 and* 2 minutes, between 2 and 12 minutes and between 12 and about 25 minutes. The variability amounted to 1.5, 2.7 and 4.0 % respectively.

As shown, the extrapolation procedure with an average regression line will involve only a moderate discrepancy as compared with the more exact method with individual regression lines, provided the experimentally derived maximal performance time falls within the time limits studied. In the use of estimated values of $PWC_{max\,6}$ for calculation of reproducibility the error of divergent individual inclination will thus *be relatively small.*

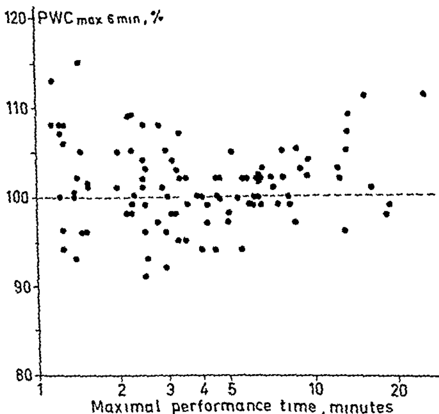


Fig 9 Error induced by calculation of PWC max 6 from one maximal performance as compared with the value derived from repeated work periods

In each subject the work intensity sustainable during 6 minutes (PWC$_{max\,6}$) was calculated with the aid of the individual regression line of 2—5 load time values For each of the performances the PWC$_{max\,6}$ was furthermore estimated according to the average relationship between work load and maximal performance time In the graph the latter values are expressed as a percentage of the more exact figures derived from repeated work periods

For the capacity of exhausting work during a maximum of 6 minutes (PWC$_{max\,6}$) as estimated from duplicate performances the variability was found to be 1 1 % of the mean value The figures of the second work period were on average slightly although not significantly higher than those of the first performance ($n = 36$ $\bar{x}_1 = 1\,165$ $\bar{x}_2 = 1\,183$ *diff* 18 kpm/min $P = 0\,3$—$0\,2$)

The experimental values of work load and corresponding maximal performance time are plotted in Fig 10 in the form of a nomogram It is possible to read estimated PWC$_{max\,6}$ directly from each experimental point The average inclination found in Series D is here used for the transformation

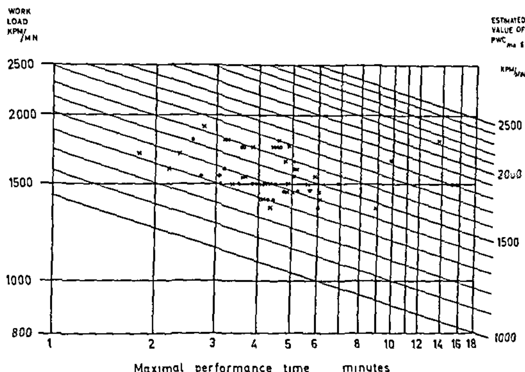


Fig 10 Work loads and maximal performance times from reproducibility studies on exhausting work

Series E $n = 36$

Experimental values of work load and maximal performance time are given in a double logarithmic graph The oblique lines of the nomogram illustrate the average relation between work load and maximal performance time established in Series D The estimated $PWC_{max\,5}$ may here be read directly from each experimental point

Symbols 1st performance o 2nd performance x

Circulatory adaptation in exhausting work of varying duration

Final heart rate after maximal performances of varying duration

The pulse reaction was studied in a series of experiments in which each individual had to work on different loads until complete exhaustion. Two different subject groups were investigated, viz. a group of civilian volunteers and a group of ordinary military conscripts.

The heart rate was recorded by Ecg, the final rate being measured during 10 heart cycles immediately after termination of work.

Comparisons between heart rates calculated before and after the end of performances had shown only minor differences. The post-exercise values were thus on average somewhat lower than the pre-termination rates ($n = 96$, $\bar{x}_1 = 179.5$, $\bar{x}_2 = 177.0$, $diff. = 2.5$ beats/min = 1.4 % of the first value). For shorter performances as compared with longer ones this difference was of about the same magnitude (duration range 45—5.59, $diff. = 2.8$ beats/min, duration range 6—18, $diff. = 2.1$ beats/min). The use of either value will therefore be equivalent for the purpose of the present investigation.

In the group of civilian volunteers (Series F) each subject participated in a series of exhausting performances with different loads. For 28 individuals final pulse rates were recorded in a total of 125 work periods.

In the group of conscripts (Series D) each man had to work on two different loads during the same day with an interval of about 4 hours. On a following day the procedure was reversed. For the present study only the results from the first work period of each day were used. Data were collected from 43 individuals. The work intensities chosen corresponded to maximal performance times of between about 1 and 18 minutes.

In Fig. 11 the heart rates recorded immediately after termination of work are put in relation to the maximal performance times. By relating the pulse values to maximal work times instead of work loads the data were better adjusted to individual capabilities. For comparison the pulse rates were also recorded after maximal work with graded loads. (See page 61.)

As can be seen, the heart rates recorded immediately after exhausting work showed characteristic differences corresponding to the duration of the work

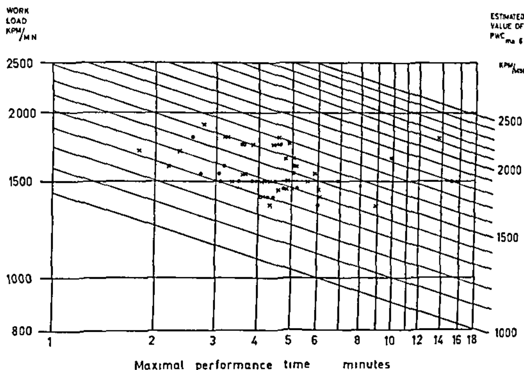


Fig 10 Work loads and maximal performance times from reproducibility studies on exhausting work

Series E $n = 36$

Experimental values of work load and maximal performance time are given in a double logarithmic graph The oblique lines of the nomogram illustrate the average relation between work load and maximal performance time established in Series D The estimated $PWC_{max\,6}$ may here be read directly from each experimental point

Symbols 1st performance o 2nd performance x

Circulatory adaptation in exhausting work of varying duration

Final heart rate after maximal performances of varying duration

The pulse reaction was studied in a series of experiments in which each individual had to work on different loads until complete exhaustion Two different subject groups were investigated viz a group of civilian volunteers and a group of ordinary military conscripts

The heart rate was recorded by Ecg the final rate being measured during 10 heart cycles immediately after termination of work

Comparisons between heart rates calculated before and after the end of performances had shown only minor differences The post-exercise values were thus on average somewhat lower than the pre termination rates (n = 86 $\bar{x}_1$ = 179 5 $\bar{x}_2$ = 177 0 *diff* = 2 5 beats/min = 1 4 % of the first value) For shorter performances as compared with longer ones this difference was of about the same magnitude (duration range 45 —5 59 *diff* — 2 8 beats/min duration range 6 —18 *diff* = 2 1 beats/min) The use of either value will therefore be equivalent for the purpose of the present investigation

In the group of civilian volunteers (Series F) each subject participated in a series of exhausting performances with different loads For 28 individuals final pulse rates were recorded in a total of 125 work periods

In the group of conscripts (Series D) each man had to work on two different loads during the same day with an interval of about 4 hours On a following day the procedure was reversed For the present study only the results from the first work period of each day were used Data were collected from 13 individuals The work intensities chosen corresponded to maximal performance times of between about 1 and 18 minutes

In Fig 11 the heart rates recorded immediately after termination of work are put in relation to the maximal performance times By relating the pulse values to maximal work times instead of work loads the data were better adjusted to individual capabilities For comparison the pulse rates were also recorded after maximal work with graded loads (See page 61)

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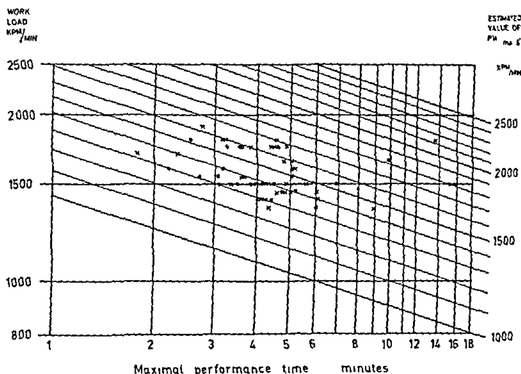


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Symbols 1st performance o, 2nd performance x

$diff = 10.8$ beats/min $P < 0.001$***) The final pulse value corresponding to a mean duration of 6.1 minutes was furthermore compared with the pulse level attained after graded exhausting work. The latter lasted on average for about 26 minutes. This difference was however not statistically significant ($n = 25$, $\bar{x}_2 = 181.1$, $n_3 = 51$, $\bar{x}_3 = 184.3$, $diff = 3.2$ beats/min, $P = 0.2$—0.1).

In Series F the examinees as mentioned took part in several exhausting performances. For each individual as well as for the entire material the experimental points could be connected to a curve of characteristic shape. The level of this curve was found to vary between individuals as well as between subject groups. (See Fig. 16.) The group of civilian volunteers was divided into three partly overlapping subgroups (g—i). It was here possible to calculate intraindividual differences of maximal heart rates attained after strenuous work of varying duration. The results of this study appear in Table 11.

Table 11 Final heart rates in Series F divided into subgroups

Subgroup	n	Maximal performance time Range Min	Mean value Min	Mean value sec	Mean heart rate beat per minute	Diff b/min	P
g	18	0—3	1	57	176	+ 8	<0.001
		3—6	4	23	184		
h	21	3—6	4	17	184	+ 4	0.001—0.01 *
		6—18	10	20	188		
i	25	6—18	10	00	189	+ 2	0.3—0.2
		19—34†	27	25	191		

† Graded maximal work

As shown above final heart rates were significantly lower after exhausting performances of short duration—2 to 4 minutes—than after longer work periods. On the other hand no significant difference was established for maximal pulse values after strenuous work lasting about 10 minutes and those after the more lengthy work in the graded work test.

In order to eliminate interindividual variability the data of Series F were also expressed in relative terms. For each individual the heart rate attained after the graded maximal test was denoted as 100 per cent. The rates recorded after the shorter work periods were expressed as a percentage of the value mentioned. The results of these calculations are shown in Fig. 12.

The findings as regards the relation between final heart rate and duration of work showed good correspondence within both of the subject groups investigated.

In summary the present results indicate that a constant or nearly constant final pulse level is first attained after maximal work periods exceeding 6

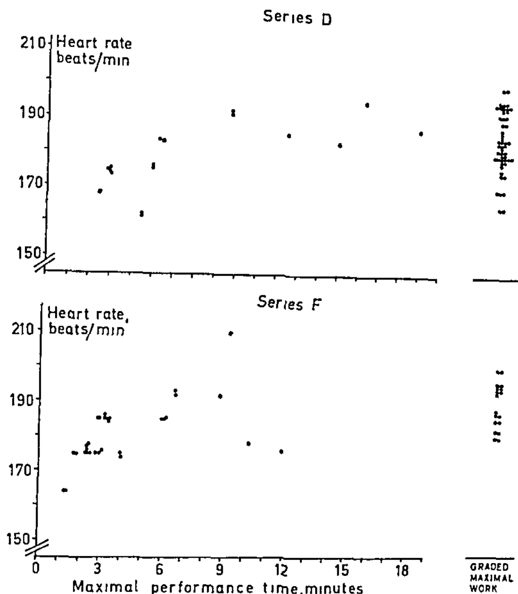


Fig 11 Final heart rates after exhausting work of varying duration in a group of 19/20 year old conscripts (D) and in a group of civilian volunteers (F)

The work loads were chosen to give maximal performances with a duration of between 1 and 18 minutes On the right are given the final heart rates for exhausting work with work loads increased stepwise

For Series D where each individual was represented by two such performances means were computed for groups of experimental points corresponding to average maximal performance times of 3 0 and 6 1 minutes respectively The difference between the mean final heart rates was here statistically highly significant ($n_1 = 27$ $\bar{x}_1 = 170\ 3$ $n_2 = 25$ $\bar{x}_2 = 181\ 1$

$diff = 10\ 8$ beats/min $P < 0\ 001$***) The final pulse value corresponding to a mean duration of 6 1 minutes was furthermore compared with the pulse level attained after graded exhausting work The latter lasted on average for about 26 minutes This difference was however not statistically significant ($n_2 = 25$ $\bar{x}_2 - 181\ 1$ $n_3 - 51$ $\bar{x}_3 = 184\ 3$ $diff = 3\ 2$ beats/min $P = 0\ 2—0\ 1$)

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g	18	0—3 3 6	1 4	57 23	176 184	+ 8	<0 001 *
h	21	3—6 6—18	4 10	17 20	184 1[illegible]	+ 4	0 001— 0 01
i	25	6—18 19—34†	10 27	00 2[illegible]	189 191	+ 2	0 3—0 2

† Graded maximal work

As shown above final heart rates were significantly lower after exhausting performances of short duration—2 to 4 minutes—than after longer work periods On the other hand no significant difference was established for maximal pulse values after strenuous work lasting about 10 minutes and those after the more lengthy work in the graded work test

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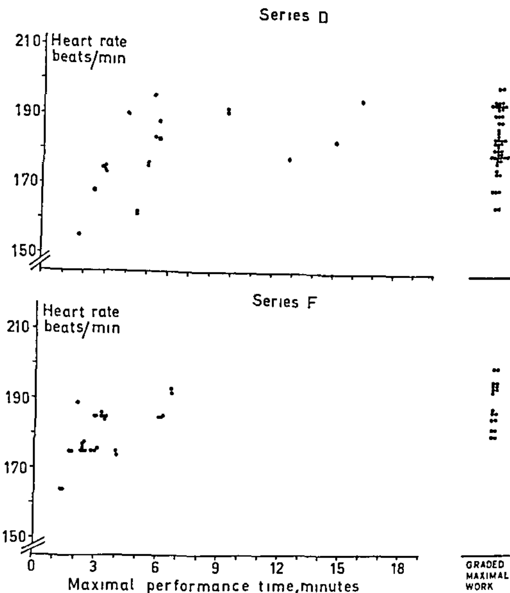


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During each work period the heart rate was continuously recorded by Ecg and the maximal oxygen uptake determined by the Douglas bag method For technical details regarding the determination of maximal oxygen uptake see page 61

In each individual the peak oxygen uptake was compared for exhausting performances of shorter and longer duration In some cases means of two determinations were utilized The results appear in Fig 13

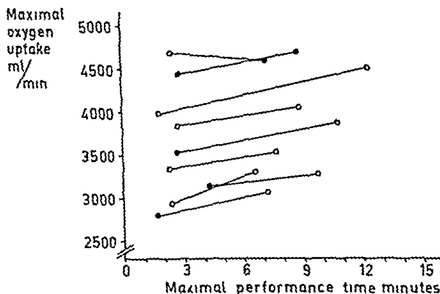


Fig 13 Maximal oxygen uptake during exhausting work of varying duration
Series F civilian volunteers $n = 9$
Unfilled circles denote experimental values from one maximal performance Filled symbols denote mean values from two maximal performances with the same or almost the same relative work load

As shown higher values were on average attained in exhausting performances of longer duration than in those of shorter duration Comparison between mean maximal oxygen volumes corresponding to average work durations of 2 39 and 8 46 thus showed a statistically significant difference ($\bar{x}_1 = 3\,628$ $\bar{x}_2 = 3\,863$ $diff = 235$ ml/min $P = 0.01$—0.001**)

Similarly the maximal pulmonary ventilation was compared for work periods of varying duration The results are given in Fig 14

Here too higher values were recorded after longer performances The difference between ventilation volumes corresponding to work durations of 2 39 and 8 46 was however not statistically significant ($\bar{x}_1 = 99\,76$ $\bar{x}_2 = 108\,11$ $diff = 8\,35$ l/min $P = 0\,1$—$0\,05$)

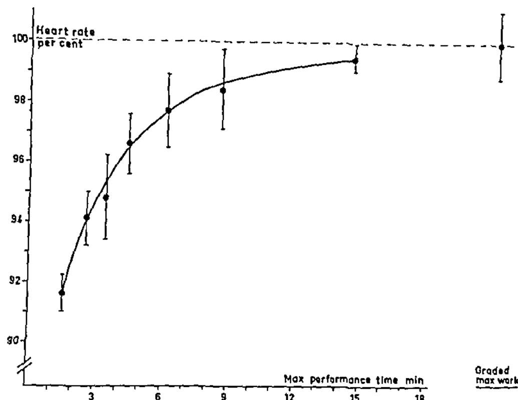


Fig 12 Final heart rates after exhausting work of varying duration in 28 civilian volunteers

The values are given as a percentage of the heart rate attained after graded maximal work Symbols denote $\bar{x} \pm s_{\bar{x}}$

minutes In performances of longer duration also a slightly higher heart rate was observed The differences were however small and statistically insignificant

Peak oxygen uptake and maximal pulmonary ventilation in exhausting work of varying duration

The above mentioned findings of divergent final heart rates after maximal performances of different duration encouraged an extension of the experimental series to include determinations of maximal oxygen uptake

In 9 individuals (Series Γ_1) the peak oxygen uptake was determined during maximal work periods of varying intensity After initial assessment of the maximal working capacity the subjects had to participate in two or more additional performances In these the work loads were chosen to give alternatively short or long maximal work periods To avoid the influence of accidental training effects here too the sequence of work loads was varied

During each work period the heart rate was continuously recorded by Ecg and the maximal oxygen uptake determined by the Douglas bag method For technical details regarding the determination of maximal oxygen uptake see page 61

In each individual the peak oxygen uptake was compared for exhausting performances of shorter and longer duration In some cases means of two determinations were utilized The results appear in Fig 13

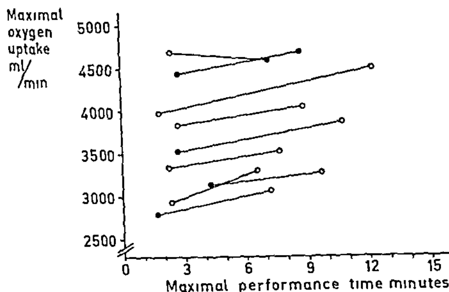


Fig 13 Maximal oxygen uptake during exhausting work of varying duration
Series F civilian volunteers $n = 9$
Unfilled circles denote experimental values from one maximal performance Filled symbols denote mean values from two maximal performances with the same or almost the same relative work load

As shown higher values were on average attained in exhausting performances of longer duration than in those of shorter duration Comparison between mean maximal oxygen volumes corresponding to average work durations of 2 39 and 8 46 thus showed a statistically significant difference ($\bar{x}_1 = 3\ 628$ $\bar{x}_2 = 3\ 863$ $diff = 235$ ml/min $P = 0\ 01 - 0\ 001^{**}$)

Similarly the maximal pulmonary ventilation was compared for work periods of varying duration The results are given in Fig 14

Here too higher values were recorded after longer performances The difference between ventilation volumes corresponding to work durations of 2 39 and 8 46 was however not statistically significant ($\bar{x}_1 = 99\ 76$ $\bar{x}_2 = 108\ 11$ $diff = 8\ 35$ l/min $P = 0\ 1 - 0\ 05$)

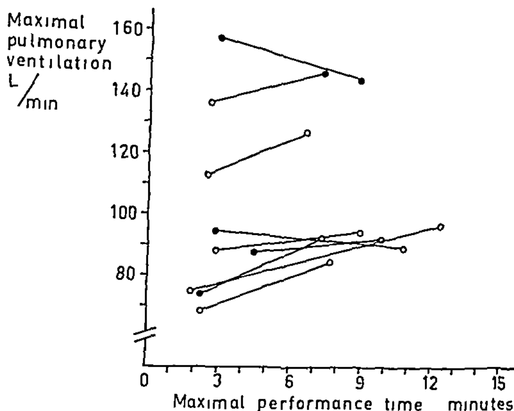


Fig 14 Maximal pulmonary ventilation during exhausting work of varying duration

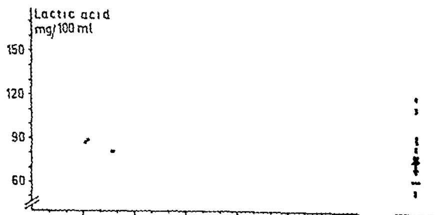
Series F civilian volunteers $n = 9$
Unfilled circles denote experimental values from one maximal performance Filled symbols *denote mean values from two maximal performances with the same or almost the same relative work load*

Final concentration of lactic acid after exhausting work of varying duration

To estimate the degree of anaerobic work in exhausting performances of varying duration, the final lactic-acid concentration in blood was determined in two groups of subjects These investigations were carried out in close connection with the above mentioned studies on final heart rate In all cases the blood samples were taken during the first few seconds after termination of the maximal work

One group consisted of ordinary conscripts (Series D) whereas the other group was composed of civilian volunteers (Series F) The work load was chosen to give maximal performances of varying duration For Series D the final lactic acid concentration was determined in 37 individuals each participating in one maximal performance For Series F sampling was performed

in 21 individuals each participating in several performances—number of determinations 80 After the graded maximal test samples were secured from 37 subjects in Series D and 16 subjects in Series F The results are given in Fig 15



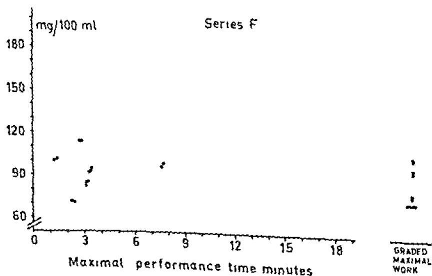


Fig 15 Final lactic acid concentration aft exhausting work of varying duration in a group of 19/20 year-old conscripts (D) and in a group of civilian volunteers (F)

The work loa is were chosen to give maximal performances of a duration between about 1 and 18 minutes In addition the final lactic-acid concentrations are et out for exhaust ing work with work loads increased stepwise

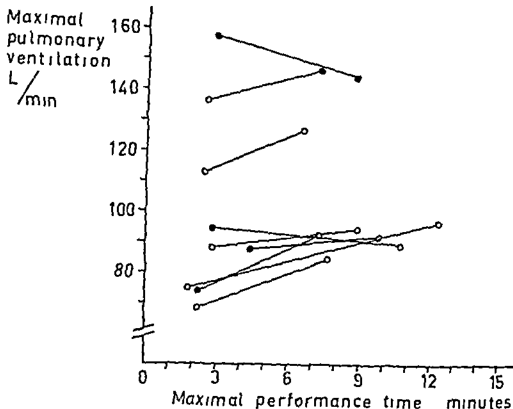


Fig 14 Maximal pulmonary ventilation during exhausting work of varying duration

Series F civilian volunteers $n = 9$
Unfilled circles denote experimental values from one maximal performance Filled symbols denote mean values from two maximal performances with the same or almost the same relative work load

Final concentration of lactic acid after exhausting work of varying duration

To estimate the degree of anaerobic work in exhausting performances of varying duration the final lactic acid concentration in blood was determined in two groups of subjects These investigations were carried out in close connection with the above mentioned studies on final heart rate In all cases the blood samples were taken during the first few seconds after termination of the maximal work

One group consisted of ordinary conscripts (Series D) whereas the other group was composed of civilian volunteers (Series F) The work load was chosen to give maximal performances of varying duration For Series D the final lactic acid concentration was determined in 37 individuals each participating in one maximal performance For Series F sampling was performed

During work at high intensities the cardiac output may not reach the same magnitude as during performances with lower loads. Especially when maximal working time is reduced below 3 minutes the highest attainable cardiac output seems to decrease significantly. At these high work loads the central circulatory capacity is evidently not used to its full extent.

Peak oxygen uptake and maximal pulmonary ventilation. The level of oxygen uptake during physical exercise is a function of several mutually interrelated factors including pulmonary ventilation, alveolar gas exchange, cardiac output, peripheral circulatory adjustment and arteriovenous difference. As has been shown by a number of investigators the metabolic rate during steady state work is roughly linearly correlated with the heart rate (29, 129). Furthermore, in studies using catheterization technique a high correlation has been established between oxygen consumption on the one hand and cardiac output as well as oxygen utilization on the other (70). For pulmonary ventilation also a similar although—especially in work of high intensities—less pronounced connection with oxygen uptake has been noted (7).

Of the factors mentioned the cardiac output seems to be the most important component function determining the magnitude of oxygen consumption during physical exercise (97).

As mentioned, a higher oxygen uptake was observed in more lengthy exhausting work (mean performance time 8 minutes and 16 seconds) than in shorter periods (mean performance time 2 minutes and 39 seconds).

In the interpretation of results the close association between oxygen supply and circulatory functions has to be taken into account. The final heart rate and the peak oxygen uptake may both approximately reflect the magnitude of cardiac output attained during exhausting work. The present results would thus seem to support the conclusions drawn from the studies of final heart rate.

Besides the function of oxygen transporting organs the degree of peripheral oxygen utilization may, however, also influence the magnitude of oxygen uptake. An increased oxygen extraction in the muscles would here theoretically be able to compensate for the incomplete adaptation of the central circulation. On the other hand, the present results do not support the hypothesis that such compensatory mechanisms exist.

In summary, the studies reported suggest that in maximal performances of short duration the oxygen supply capacity is generally not fully utilized. The probable explanation for this seems to be that the time of work may be too short for maximal circulatory adjustment.

As can be seen, the final lactic acid concentrations show a considerable scattering between high and low values in both subject groups investigated For short intensive performances as well as longer, easier ones a considerable elevation of lactic-acid concentrations was observed as compared with the levels generally recorded at rest

A comparison between the concentrations after exhausting performances of varying duration showed similar differences within both experimental groups The highest mean value was found after performances of a duration approximating 10 minutes For shorter as well as longer work periods lower mean values were recorded The differences observed were however not statistically significant in any of the subject groups investigated

In Table 12 mean intraindividual differences are given for three partially overlapping subgroups of Series F (k—m)

Table 12 Final lactic acid concentrations in Series F divided into subgroups

Subgroup	n	Maximal performance time Range Min	Mean value Min	sec	Mean lactic acid conc mg/100 ml	Diff mg/100 ml	P
k	11	0—3	1	53	95 7	+ 11 1	0 3—0 2
		3—6	4	18	106 8		
l	12	3—6	4	02	101 0	+ 7 4	0 4—0 3
		6—18	10	49	108 4		
m	14	6—18	9	29	106 8	— 11 5	0 1—0 05
		19—34†	27	25	95 3		

† Graded maximal work

Discussion

Final heart rate The magnitude of cardiac output is determined by the stroke volume on the one hand and the heart rate on the other The changes in cardiac output during adaptation to work are mainly due to the increased heart rate the augmentation of stroke volume being less pronounced The latter seems predominantly to occur during work in the sitting position being confined to the period shortly after the commencement of exercise The mechanism of this increase has been explained by a redistribution of blood from the leg vasculature to the central circulation (2 19 70 81 116)

In the studies reported the final heart rate was found to be on average lower in short periods of exhausting work than in performances of longer duration If the stroke volume is assumed to be the same in both events the observations made in the present study may have the following significance

The highest attainable oxygen uptake heart rate and lactic acid concentration after exhausting work of varying duration has earlier been studied by Åstrand & Saltin (13) Each performance was here preceded by a 10 minutes period of warming up using a work intensity corresponding to 55 % of the maximal oxygen uptake No significant differences were found for maximal oxygen uptake and heart rate if the length of work on the highest load was varied between 1 and 8 minutes Similar results were obtained for maximal lactic acid concentration whereas ventilatory volumes showed significantly higher values after the short heavy performances

In the study just cited the purpose of investigation as well as the design of the experiments differed in essential respects from those of the present study The preliminary exercise may have contributed to a better circulatory adjustment in performances of high intensity than was attained in the present investigation where work was initiated directly with a high work load This may account for the disparity of results Other factors such as divergence in the physical condition of the material may also be of importance in explaining the discrepancy

6—20 ,614

Final lactic-acid concentration In earlier studies of exhausting performances the lactic acid concentration has been used as an approximate measure of anaerobic work The basis for these estimations has been the relation between oxygen debt and lactic acid formation (11, 16 37, 44, 64, 79) In the present investigation it was considered to be of interest to compare the degree of anaerobic work in exhausting work of varying duration

In two subject groups studied in this respect the lactic acid concentration was measured in finger-prick blood immediately after the conclusion of the maximal work A large scattering of values as well as moderate disparities were established for final lactic acid after exhausting performances of varying duration The differences were however not statistically significant in either of the groups

In evaluating the results it should be remembered that besides muscular hypoxia several other factors such as forced hyperventilation and elevated pyruvate concentration, may influence the lactic acid levels (72 73) This may account for the large intra- as well as interindividual variation observed Furthermore in the performances of short duration the values recorded may be somewhat too low, owing to the lag of circulation from the muscles to the sampling site If the sampling is delayed however another error will be induced, viz metabolism of lactic acid by the resting muscles after work

Even if these possible errors are taken into consideration the present results seem to indicate that the degree of anaerobic work will be of about the same order of magnitude in exhausting performances of varying duration (between about 1 and 25 minutes)

From the present findings it may be suggested that the following sequence of events occurs in maximal exertions of varying duration In exhausting work with high work loads lasting for 2—3 minutes the large energy demands of the muscles will only to a small extent be met by the oxygen available anaerobic mechanisms have to provide the greater part of the energy supply During the course of the work a concentration of anaerobic metabolites will rapidly accumulate leading to a termination of work before maximal circulatory adaptation is attained If on the other hand the load is put lower allowing the work to continue for e g 6—10 minutes the energy demands of the working muscles will be more moderate The oxygen supplied by the circulatory system will be able to balance the energy demands more effectively As the work continues however anaerobic metabolism will here too, give rise to an accumulation of metabolic products although at a more gradual rate The interval before termination of work is provoked will apparently be sufficiently long for practically maximal adjustment of the circulatory system to occur

Table 13 Final heart rate and lactic acid concentration after exhausting work

Reference No	Author	Category	n	Age	Type of work	Maximal heart rate beats min	Maximal lactic acid mg 100 ml
40	Dill D B & Brouha L	men	14	20—36	running on treadmill	195	—
109	Robinson S	men	11	20—29	running on treadmill	189	89
96	Metheny E L *et al*	college men	30	19—23	running on treadmill	194	119
[illegible]6	Brouha L & Heath C	college men	1[illegible]0	1[illegible]—22	running on treadmill	193	—
30	Christensen E & Högberg P	school boys	21	14—16	skiing 5 km	23[illegible]	—
11 A	Åstrand P O	college men	42	20—33	running on treadmill and cycling	194	112
B		college women	44	20—25	running on treadmill and cycling	198	103
118	Slonim N B Gillispie D & Harold W	naval aviation cadets	50	18—25	running on treadmill	188	—
1[illegible]3	Döbeln W v Engström C G	pilot candidates	21	18	cycling	204	107
	Malmström G & Ström C	pilots	21	40	cycling	185	108
9	Åstrand I	women mostly house wives	8	[illegible]0—[illegible]9	cycling	187	121

As shown above the level of final heart rate after exhausting effort is partly dependent upon the duration of work When comparing maximal heart rates in different subject groups therefore the length of performance has to be taken into consideration In Fig 16 final pulse rates in different series after equivalent work were compared

It was here found that for short as well as longer exhausting performances —at least for durations below ½ hour—the volunteers tended to reach higher final pulse levels than did average conscripts

For one of the subject groups viz Series F the graded work test could not be applied because the examinees could be available only during

Maximal physical performances in different civilian and military subject groups with special regard to final heart rates and lactic-acid concentrations attained

As pointed out above, divergent final pulse levels may be attained in physical exertions of different kinds The concept of maximal pulse should therefore *not be used without definition of the type of exercise as well as the length of work*

Besides intraindividual variations according to the character of the work considerable interindividual differences are encountered At present, not all the factors determining the individual level are known However, psychological mechanisms above all the motivation may be assumed to play an important role in limiting maximal performances Motivation is here defined *as the willingness of the subject to perform physical work until complete exhaustion*

To elucidate the importance of the individual attitude some results of earlier investigators are summarized In the present study different subject groups with divergent psychological conditions are also studied in this respect

In Table 13 nine studies, quoted from the literature are compared in *regard to character of material, size of subject group age type of work* maximal heart rate and lactic acid concentration As can be seen the composition of the material varies from one study to another Mostly the subjects are college students or military candidates Both in regard to physical capacity and desire to do well these may be assumed to surpass ordinary individuals As a rule they exhibit high values of maximal heart rate as well as of lactic acid concentration after maximal exertions Comparison with subject groups of more average composition shows that the latter generally have lower final levels (cf refs 9 and 11 B)

In the present investigation four subject groups were studied in regard to pulse rate recorded immediately after exhausting performances Two of them were conscript groups *ordered to undergo examination one group* consisting of non selected individuals (Series D) the other being selected at random (Series B_1) A third group was composed of *military volunteers* (Series E) and a fourth consisted of civilian volunteers (Series F)

The final heart rate after the graded work test performed until exhaustion was furthermore compared between Series F (civilian volunteers) and Series D (ordinary conscripts) Here too the voluntary subjects showed higher pulse values than did the individuals ordered to undergo examination ($\bar{x}_D = 184.4$ $\bar{x}_F = 191.2$ *diff* $= 6.8$ beats/min P^{**}) For Series F compared with Series B_1 (randomly selected conscripts) the difference was probably significant ($\bar{x}_B = 186.5$ $\bar{x}_F = 191.2$ *diff* $= 4.7$ beats/min P^{*})

Between the two conscript groups exhibiting a similar psychological atti tude no significant difference was found as regards maximal heart rates

Besides final heart frequency some other biological data were compared between the different categories investigated As regards age the civilian volunteer were on average six years older than the military conscripts They were furthermore somewhat heavier and revealed significantly higher values of estimated subcutaneous fat than members of the other groups The PWC_{170} was of about the same magnitude as for the conscripts The values of maximal working capacity ($PWC_{max\ 6}$) were however slightly higher than for the men ordered to undergo examination (*Diff* P^{*}) A higher maxi mal physical working capacity was similarly established for the military volunteers as compared with the ordinary conscripts (*Diff* P^{*}) See Table 1 As far as recorded data are concerned no other significant differences were observed

A comparison of the present results with those cited from the literature was furthermore performed With reservation for differences in respect of investigational techniques the maximal pulse of randomly selected conscripts seems to be of the same order of magnitude as for the subjects investigated by for instance Slonim *et al* (118) Compared with other mostly voluntary subject groups the final heart rate appeared to be lower at least for this type of work

Beside maximal heart frequency the lactic acid concentration was determined in two groups of subjects viz Series D (ordinary conscripts) and Series F (civilian volunteers) Since no significant differences were found in concentrations after exhausting performances of varying duration, means were computed for all final lactic acid values of the separate groups The volunteers here showed a significantly higher average concentration after maximal work than did the ordinary conscripts ($\bar{x}_D = 81.4$ $\bar{x}_F = 10?.9$ *diff* 21.1 mg per 100 ml $P < 0.001^{***}$)

Discussion Four subject groups of different composition were investigated in the present study Military groups of ordinary conscripts as well as indi viduals with a pronounced interest in high test results were examined In

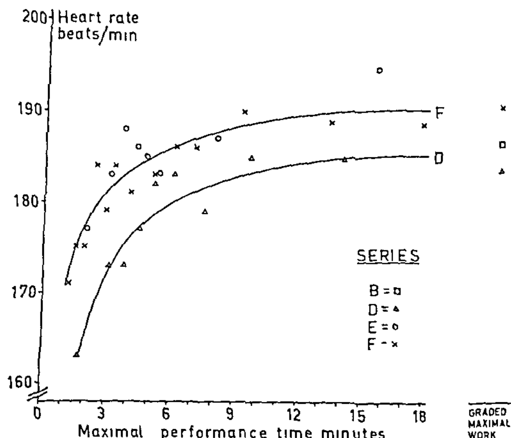


Fig 16 Final heart rates after exhausting work of varying duration A comparison of maximal heart rate in subject groups of different composition

Series B (□) = randomly selected conscripts
" D (△) = non selected conscripts
" E (○) = military volunteers
" F (×) = civilian volunteers

For subject groups D E and F the symbols in the left part of the diagram represent means of 10 experimental values The resulting curves are drawn freehand for Series D and F The symbols on the right denote means of whole subject groups expressing average final pulse after graded maximal work

two days used for duplicate maximal tests with constant work loads Here final pulse values are compared with those of Series D in both cases *after* exhausting performances with a mean duration of 3 minutes and 55 seconds It was found that the volunteers attained a significantly higher heart rate than did the ordinary conscripts The difference of comparable maximal pulse frequencies was found to be statistically highly significant ($n_D = 55$ $n_E = 60$ Maximal performance time range 0—6 min mean value 3 minutes 55 seconds Final pulse values $\bar{x}_D = 174$ $\bar{x}_F = 181$ *diff* = 10 beats/min p^{***})

ical characteristics mentioned A reliable criterion of the individual motivation would seem to be one important prerequisite for correlation studies of this kind It is possible that psychological investigations will be able further to elucidate the association between final heart rate and lactic acid concentration after strenuous work on the one hand and motivation on the other

addition, one civilian group was studied representing a selection of people with a high degree of motivation

Subject groups selected at random—as for instance the military conscripts in Series B_1—may *a priori* be assumed to contain individuals with a variety of attitudes to physical exertion Compared with volunteers they cannot on average be expected to have the same degree of motivation

In the present study, factors other than the natural willingness to participate in exhausting work were as far as possible reduced Thus all the conscripts were informed about the scientific purposes of the investigation, as described on page 18

As mentioned a higher mean final heart rate was observed in the group of civilian volunteers than in the two groups of ordinary conscripts According to the findings of earlier investigators the maximal heart frequency generally decreases with advancing age (9 109) Thus a slightly lower value would have been expected instead The discrepancy regarding weight and estimated subcutaneous fat was rather small and probably cannot be related to the circulatory data discussed

The two groups of ordinary conscripts on the one hand and the military and civilian volunteers on the other were selected in different ways As the different motivation seemed to be the dominating factor separating these two categories of examinees this quality may reasonably be connected with the disparity in final heart rates

The finding of a generally higher final lactic acid level in the group of civilian volunteers than in the conscripts would indicate that the former were able to contract a larger oxygen debt than were the conscripts Here again the difference of psychological attitude may account for the discrepancy

In the subject groups reported on by earlier investigators the values of lactic acid observed at the end of exhausting work were generally higher than those recorded in the present series It may be pointed out that the actual levels were found in samples taken immediately after the termination of work whereas the values of earlier authors as a rule represent maximal concentrations reached 3—5 minutes after termination of work This may explain the differences observed

Comparisons between groups of subjects thus strongly suggested that there is a close connection between the height of final heart rate and lactic acid concentration after exhausting work on the one hand and motivation on the other

For single individuals however it was not possible to draw any definite conclusions regarding the association between the physiological and psycholog

Table 14 B Correlation between PWC_{max} (x) and PWC (y) $\dot{V}o_2 max$ (y)

Series	Determination	$PWC_{max\,1}$	$PWC_{max\,3}$	$PWC_{max\,6}$	$PWC_{max\,10}$	$PWC_{max\,15}$
F_1	$\dot{V}o_2 max$	$r = 0.83$	$r = 0.91$ *	$r = 0.94$ *	$r = 0.95$***	$r = 0.95$ *
$n = 9$	$\bar{y} = 3\,874$	$s = 381$	$s_e = 237$	$s_e = 237$	$s_e = 216$	$s_e = 207$
F	PWC_{170}	$r = 0.39$	$r = 0.46$	$r = 0.49$	$r = 0.50$	$r = 0.49$*
$n = 28$	$\bar{y} = 1\,086$	$s = 156$	$s = 151$	$s_e = 148$	$s = 147$	$s_e = 148$

Note r = correlation coefficient s_e = standard deviation of residuals Figures of $\dot{V}o_{max}$ are given in ml/min values of PWC_{170} are given in kpm/min

metric strength was recorded with the aid of the strain gauge method described earlier As an expression of general muscle strength there was here used the standardized muscle factor according to the definition reported

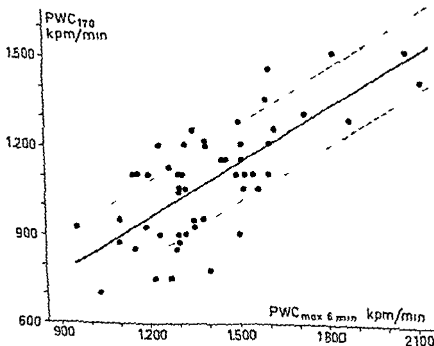


Fig 17 Correlation between $PWC_{max\,6}$ and PWC_{170}

Series D n = 52

Scatter diagram illustrating the relation between the working intensity sustainable during a maximum of 6 minutes and the working intensity at pulse 170 in circulatory steady state Broken lines denote ± standard error of estimate

CHAPTER XII

Relation between different methods for assessment of physical capability

To establish the significance of different expressions of physical working capacity the present results were related to those of some other methods.

Investigations were carried out in which besides maximal physical working capacity (PWC_{max}), the working capacity at a pulse of 170 (PWC_{170}) and maximal oxygen uptake ($V_{O_2 max}$) were determined. The latter was defined as the peak oxygen uptake attained during exhausting work of sufficient duration to give a maximal circulatory adaptation.

The material used consisted of the group of civilian volunteers described earlier (Series F) and the two groups of ordinary non selected conscripts (Series C and D). The determinations of $V_{O_2 max}$ were performed in 9 members of the voluntary group (Series F_1).

In Series F the time load curve was established for each individual. From the regression equation different expressions of the maximal working capacity were calculated according to the duration of work. In the conscript groups the capacity corresponding to a maximal performance time of 6 minutes was estimated by intra- or extrapolation from two or occasionally three experimental values ($PWC_{max\ 6}$).

The above mentioned expressions of maximal working capacity were studied in relation to PWC_{170} and partly also to $V_{O_2 max}$. The results of these correlation studies appear in Tables 11 A and B.

For one of the two subject groups composed of non selected military conscripts (Series D) the scatter diagram below shows regression line as well as standard error of estimate. See Fig. 17.

In two of the groups investigated (C and F) the maximal voluntary iso

Table 11 A. Relationship between $PWC_{max\ 6}$ (x) and PWC_{170} (y)

Series	n	r	P	Equation of regression line	s_e
C	58	0.67	***	$y = 0.589x + 233$	118
D	52	0.72	***	$y = 0.595x + 243$	131
F	28	0.49	**	$y = 0.372x + 523$	148

Note. Values are given in kpm/min

Table 14 B Correlation between PWCmax (x) and PWC (y) $\dot{V}o_2$max (y)

Series	Determination	$PWC_{max\ 1}$	$PWC_{max\ 3}$	$PWC_{max\ 6}$	$PWC_{max\ 10}$	$PWC_{max\ 15}$
F_1	$\dot{V}o_2$ max	$r = 0.83$	$r = 0.91^{**}$	$r = 0.94$	$r = 0.95$	$r = 0.95^{**}$
$n = 9$	$\bar{y} = 3\,874$	$s = 381$	$s_e = 237$	$s_e = 237$	$s_e = 216$	$s_e = 207$
F	PWC_{170}	$r = 0.39$	$r = 0.46^{*}$	$r = 0.49$	$r = 0.50$	$r = 0.49$
$n = 28$	$\bar{y} = 1\,086$	$s_e = 156$	$s_e = 151$	$s = 148$	$s = 147$	$s_e = 148$

Note r = correlation coefficient s = standard deviation of residuals Figures of $\dot{V}o_{2\,max}$ are given in ml/min values of PWC_{11} are given in kpm/min

metric strength was recorded with the aid of the strain gauge method described earlier As an expression of general muscle strength there was here used the standardized muscle factor according to the definition reported

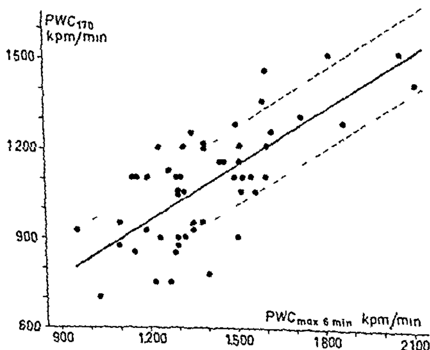


Fig 17 Correlation between $PWC_{max\,6}$ and PWC_{170}

Series D n = 52

Scatter diagram illustrating the relation between the working intensity sustainable during a maximum of 6 minutes and the working intensity at pulse 170 in circulatory steady state Broken lines denote ± standard error of estimate

on page 11 For calculation of the muscle factor of the [illegible] als the mean values as well as standard deviations [illegible] series were used

The results of correlation studies between PWCmax [illegible] one hand and the muscle factor on the other are shown [illegible]

Table 15 Correlation between PWC and muscle strength (muscle factor)

Series	$PWC_{max\,1}$	$PWC_{max\,3}$	$PWC_{max\,6}$	$PWC_{max\,10}$	[illegible]
I $n = 27$	$r = 0.60^{***}$	$r = 0.69^{***}$	$r = 0.69^{***}$	$r = 0.68^{***}$	[illegible]
C $n = 56—58$	—	—	$r = 0.31^{*}$	—	[illegible]

Note r = correlation coefficient

Discussion As shown above the correlation between PWCmax 6 and [illegible] was remarkably high The strong association between these two types of physical working capacity may perhaps be sought in the fact that [illegible] are estimated from results of maximal performances With regard to [illegible] for the small size of the material investigated the close correspondence between these methods may justify an interchangeable use of the techniques mentioned

The submaximal test of Karolinska Sjukhuset in which the work intensity is determined in relation to the pulse of 170 in relative [illegible] steady state seems to afford an objective test of physical working capacity In studies using the results from ski running as a criterion of end[illegible] a high correlation with PWC_{170} was found in a group of military conscripts (21) The character of this method makes it especially suitable for objective evaluation of the capacity of the oxygen transporting system whereas [illegible] maximal tests other factors as well may influence the results

It was therefore considered to be of great interest to study in what degree maximal tests of physical working capacity might give information about the PWC_{170} of the individual and vice versa

In one civilian and two military subject groups statistically significant correlations were found between $PWC_{max\,6}$ and PWC_{170} The degree of correlation showed moderate differences from category to category Neither of the military groups was randomly selected and consequently they were not strictly representative of the population studied As they were composed

of average conscripts some conclusions may however be drawn regarding the possibilities of prediction from one method to the other

Thus it appears that in ordinary conscripts the PWC_{170} may be predicted from $PWC_{max\,6}$ with an error of about 12 % (the standard error of estimate in Series C was 10 7 % in Series D it was 12 4 % of the mean value)

In this part of the study were also included correlations between PWC_{max} defined in different ways and PWC_{170} as well as V_{O_2max} It was found that the capacity for short heavy work correlated somewhat lower with both PWC_{170} and V_{O_2max} than did the capacity for longer work This observation may accord with the above suggested hypothesis of an incomplete circulatory adaptation in exhausting performances of a shorter duration than 6 minutes

The investigations on maximal voluntary isometric strength indicated that the correlation is of about the same degree between strength and the different expressions for maximal work capacity studied Obviously the muscle strength may be about equally important in both shorter and longer maximal performances in the duration range 1—15 minutes—at least in highly motivated individuals Between muscle strength and the physical working capacity at a pulse of 170 however the correlation was lower The latter observation was made in both series investigated

The correlation found between $PWC_{max\,6}$ on the one hand and PWC_{170} on the other was differently high in average conscripts and civilian volunteers This was also the case for the correlation between $PWC_{max\,6}$ and muscle strength although in the opposite direction Here it seems that the volunteers were using a greater part of their muscle strength in the maximal test ($PWC_{max\,6}$) than were the ordinary subjects In the latter however the cardiopulmonary function was presumably more decisive for the results obtained

In clinical application submaximal tests may be the only choice in evaluation of the physical working capacity The value obtained will here reflect especially the cardio-circulatory capacity On the other hand when a maximal test can be used it will in addition give information regarding the muscle strength and subjective motivation of the individuals In physical classification for example in selection of military personnel this may not involve any disadvantage as the latter quality is one important aspect of individual fitness for military use The latter assumption is valid only provided that the motivation at the time of examination corresponds to that in the service concerned

on page 41 For calculation of the muscle factor of the participating individuals the mean values as well as standard deviations from the corresponding series were used

The results of correlation studies between PWC_{max} and PWC_{170} on the one hand and the muscle factor on the other are shown in Table 15

Table 15 Correlation between PWC and muscle strength (standardized muscle factor)

Series	$PWC_{max\ 1}$	$PWC_{max\ 3}$	$PWC_{max\ 6}$	$PWC_{max\ 10}$	$PWC_{max\ 15}$	PWC_{170}
Γ $n = 27$	$r = 0.60$***	$r = 0.69$***	$r = 0.69$***	$r = 0.68$***	$r = 0.65$***	$r = 0.46$
C $n = 56—58$	—	—	$r = 0.34$*	—	—	$r = 0.19$

Note r = correlation coefficient

Discussion As shown above the correlation between $PWC_{max\ 6}$ and $\dot{V}o_{2max}$ was remarkably high The strong association between these two expressions of physical working capacity may perhaps be sought in the fact that both are estimated from results of maximal performances With reservation for the small size of the material investigated the close correspondence between these methods may justify an interchangeable use of the techniques mentioned

The submaximal test of Karolinska Sjukhuset in which the work intensity is determined in relation to the pulse of 170 in relative circulatory steady state seems to afford an objective test of physical working capacity In studies using the results from ski running as a criterion of endurance a high correlation with PWC_{170} was found in a group of military conscripts (24) The character of this method makes it especially suitable for objective evaluation of the capacity of the oxygen transporting system whereas in maximal tests other factors as well may influence the results

It was therefore considered to be of great interest to study in what degree maximal tests of physical working capacity might give information about the PWC_{170} of the individual and vice versa

In one civilian and two military subject groups statistically significant correlations were found between $PWC_{max\ 6}$ and PWC_{170} The degree of correlation showed moderate differences from category to category Neither of the military groups was randomly selected and consequently they were not strictly representative of the population studied As they were composed

of average conscripts some conclusions may however be drawn regarding the possibilities of prediction from one method to the other

Thus it appears that in ordinary conscripts the PWC_{170} may be predicted from $PWC_{max\ 6}$ with an error of about 12 % (the standard error of estimate in Series C was 10 7 % in Series D it was 12 4 % of the mean value)

In this part of the study were also included correlations between PWC_{max} defined in different ways and PWC_{170} as well as $V_{O_2\ max}$ It was found that the capacity for short heavy work correlated somewhat lower with both PWC_{170} and V_{O_2max} than did the capacity for longer work This observation may accord with the above-suggested hypothesis of an incomplete circulatory adaptation in exhausting performances of a shorter duration than 6 minutes

The investigations on maximal voluntary isometric strength indicated that the correlation is of about the same degree between strength and the different expressions for maximal work capacity studied Obviously the muscle strength may be about equally important in both shorter and longer maximal performances in the duration range 1—15 minutes—at least in highly motivated individuals Between muscle strength and the physical working capacity at a pulse of 170 however the correlation was lower The latter observation was made in both series investigated

The correlation found between $PWC_{max\ 6}$ on the one hand and PWC_{170} on the other was differently high in average conscripts and civilian volunteers This was also the case for the correlation between $PWC_{max\ 6}$ and muscle strength although in the opposite direction Here it seems that the volunteers were using a greater part of their muscle strength in the maximal test ($PWC_{max\ 6}$) than were the ordinary subjects In the latter however the cardiopulmonary function was presumably more decisive for the results obtained

In clinical application submaximal tests may be the only choice in evaluation of the physical working capacity The value obtained will here reflect especially the cardio-circulatory capacity On the other hand when a maximal test can be used it will in addition give information regarding the muscle strength and subjective motivation of the individuals In physical classification for example in selection of military personnel this may not involve any disadvantage as the latter quality is one important aspect of individual fitness for military use The latter assumption is valid only provided that the motivation at the time of examination corresponds to that in the service concerned

on page 41 For calculation of the muscle factor of the participating individuals the mean values as well as standard deviations from the corresponding series were used

The results of correlation studies between PWC_{max} and PWC_{170} on the one hand and the muscle factor on the other are shown in Table 15

Table 15 Correlation between PWC and muscle strength (standardized muscle factor)

Series	$PWC_{max\ 1}$	$PWC_{max\ 3}$	$PWC_{max\ 6}$	$PWC_{max\ 10}$	$PWC_{max\ 15}$	PWC_{n}
F $n = 27$	$r = 0.60$***	$r = 0.69$***	$r = 0.69$***	$r = 0.68$***	$r = 0.65$***	$r = 0.46$*
C $n = 56$—58	—	—	$r = 0.34$*	—	—	$r = 0.19$

Note r = correlation coefficient

Discussion As shown above the correlation between $PWC_{max\ 6}$ and $\dot{V}o_{2\ max}$ was remarkably high The strong association between these two expressions of physical working capacity may perhaps be sought in the fact that both are estimated from results of maximal performances With reservation for the small size of the material investigated the close correspondence between these methods may justify an interchangeable use of the techniques mentioned

The submaximal test of Karolinska Sjukhuset in which the work intensity is determined in relation to the pulse of 170 in relative circulatory steady state seems to afford an objective test of physical working capacity In studies using the results from ski running as a criterion of endurance a high correlation with PWC_{170} was found in a group of military conscripts (24) The character of this method makes it especially suitable for objective evaluation of the capacity of the oxygen transporting system whereas in maximal tests other factors as well may influence the results

It was therefore considered to be of great interest to study in what degree maximal tests of physical working capacity might give information about the PWC_{170} of the individual and vice versa

In one civilian and two military subject groups statistically significant correlations were found between $PWC_{max\ 6}$ and PWC_{170} The degree of correlation showed moderate differences from category to category Neither of the military groups was randomly selected and consequently they were not strictly representative of the population studied As they were composed

of average conscripts some conclusions may however be drawn regarding the possibilities of prediction from one method to the other

Thus it appears that in ordinary conscripts the PWC_{170} may be predicted from $PWC_{max\ 6}$ with an error of about 12 % (the standard error of estimate in Series C was 10 7 % in Series D it was 12 4 % of the mean value)

In this part of the study were also included correlations between PWC_{max} defined in different ways and PWC_{170} as well as $\dot{V}o_{max}$ It was found that the capacity for short heavy work correlated somewhat lower with both PWC_{170} and $\dot{V}o_{max}$ than did the capacity for longer work This observation may accord with the above suggested hypothesis of an incomplete circulatory adaptation in exhausting performances of a shorter duration than 6 minutes

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Summary and conclusions

Some problems pertinent to the assessment of maximal isometric muscle strength and physical working capacity were investigated

To establish the relation between the strength of single muscle groups, the muscle force in different parts of the body had to be determined For this purpose a method was designed whereby isometric strength could be measured in 22 mainly large muscle groups

The maximal contractile force was evaluated for attempted movements using certain well defined positions For adjustment of positions and for fixation a stand in the shape of a chair was used A set of strain gauge dynamometers attached to the chair were connected to a central recording instrument containing a measuring bridge and an alternating current amplifier The values were read directly in kiloponds, according to prior calibration

The technique was evaluated in regard to instrumental and biological errors Reproducibility was established for different phases of testing

The significance of strength evaluation with different methods was furthermore discussed

The method was applied to the study of the muscle strength patterns in military conscripts, civilian volunteers and two categories of élite athletes

In one conscript group the interrelationship of muscle strength was studied for different parts of the body On average the correlation between single muscle groups was high Certain muscles however were judged to be preferable in short tests of strength Reproducibility as well as accessibility for measuring had here also to be taken into consideration

A group of conscripts was followed during the nine months of basic military service Changes of muscle strength were found to be small and heterogeneous In part they could be ascribed to the effect of physical training during military service but they may also have been partly due to the fading of civilian environmental influences This was the case with the physical working capacity as well (PWC_{170}) The relative constancy of muscle strength, however may indicate that the muscular demands of military service will on the whole correspond to those of average civilian life

During the observation period the body height was stationary the body weight increased and the estimated subcutaneous fat decreased The significance of these findings is discussed in relation to those of earlier investigators

In the second part of the experimental study some problems connected with the evaluation of maximal physical working capacity were investigated

A method earlier described by Grosse Lordemann & Muller was applied The relationship between work intensity and maximal performance time suggested by these authors was confirmed in the present investigation

The reproducibility of maximal physical work was tested in a group of military conscripts taking part in duplicate performances on separate days For each time value the work intensity corresponding to a maximal performance time of 6 minutes was estimated The maximal working capacity expressed in this way showed a variation of 4 1 % of the mean value

As one aspect of maximal working capacity the circulatory adaptation was studied in exhausting work of varying duration The final heart rate and the peak oxygen uptake measured in exhausting work of short duration (2—3 minutes) were found to be significantly lower than the levels attained in longer maximal performances (6—10 minutes) For final lactic acid concentration no statistically significant differences were established The results seem to indicate that the circulatory capacity is not completely utilized in strenuous work of short duration It is only in exhausting performances lasting for more than 6 minutes that the circulatory adaptation may be maximal or nearly maximal The studies on final lactic acid may suggest that the oxygen debt contracted will be of about the same order of magnitude in maximal performances lasting between about 1 and 25 minutes

Groups of volunteers were found to reach on average higher final heart rates and lactic acid concentrations than did groups of ordinary conscripts The significance of these observations is discussed in relation to results reported by earlier investigators

A comparative study was performed of expressions of maximal working capacity (PWC_{max}) on the one hand and maximal oxygen uptake ($V_{O_2}max$) and the physical working capacity at a pulse of 170 (PWC_{170}) on the other The correlation between $PWC_{max\,6}$ and $V_O\,max$ was remarkably high a finding explained by their common character of maximal tests Between $PWC_{max\,6}$ and PWC_{170} a statistically significant although lower correlation was found This may be ascribed to the fact that these two measures probably reflect different aspects of physical working capacity Whereas the PWC_{170} will give an objective value of the capacity of the oxygen transporting system the PWC_{max} will also contain an element of motivation and muscular strength With reservation for the representativity of the material investigated prediction of PWC_{170} from $PWC_{max\,6}$ seems to involve an error of about 12 % in ordinary conscript groups

For maximal voluntary isometric muscle strength the correlation with different expressions of physical working capacity was studied and the possible significance of findings is discussed

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THE RATE OF SECRETION AND ELECTROLYTE CONTENT OF NORMAL GASTRIC JUICE

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BENGT NORDGREN

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CONTENTS

INTRODUCTION

Most of our knowledge concerning the physiology of gastric secretion has been obtained in experiments on animals This is especially true as regards quantitative studies The reason for this has undoubtedly been the greater ease with which such experimentation can be performed on animals as compared to man The need for quantitative data is great and the purpose of this investigation was therefore to study the following interrelationships in *man*

(1) The injection rate of histamine and the rate of gastric secretion
(2) The rate of gastric secretion and the concentrations of H^+ K^+ Na^+ and Cl^- in the secretion

These relationships have been studied extensively in Heidenhain pouch dogs (for references see HEINZ and ÖBRINK 1954) In man however only preliminary studies have been carried out (NORDGREN 1958) and the data about these relationships in man are rather incomplete The aim of the present study was therefore to acquire complementary data from human subjects without obvious signs of gastrointestinal or other disease For comparison similar experiments were made on dogs with nearly intact stomachs (emptied through a fistula in the abdominal wall)

Notwithstanding the fact that there is a vast literature concerning the composition of the gastric juice in man most of the studies reported were carried out with single subcutaneous doses of histamine or other stimulation procedures giving transient secretion values To interpret such non steady state values and especially to interrelate them is at present too difficult In the present study care has been taken to try and keep all parameters at steady state levels thus avoiding the difficulties introduced by time lags and adjustment lapses Details about the material and the methods used will be described in the relevant chapters

was removed by a tube placed in the mouth Before each experiment the subject had fasted for at least twelve hours but no restriction was placed on water intake Each experiment lasted about three to five hours and the subject sat leaning backwards in a comfortable chair Each subject was used for several experiments The injection procedure was the same as in the dog experiments For doses and injection volumes see Table 1 I The range of the dose of chlorpheni ramine was 5 to 16 mg and of uranine 0 06 to 0 10 gram

Analysis

Acidity

Two methods were used

(*a*) 0 1 ml of sample was diluted with 4 ml of deionized water and titrated with 10 mN NaOH with bromthymolblue as indicator (pH range 6 0–7 6) The NaOH solution was always checked daily against at least three 0 1 ml samples of 100 mN HCl (standard) The diluent water (4 ml deionized water) was also titrated on each occasion with NaOH to check that it contained no significant acidity The standard error in the acidity determination was ± 1 mN

(*b*) 1 ml of sample was titrated with Topfers mixed indicator di methylamino azo benzene end point pH 3 5 for the free acidity and phenolphthalein end point pH 8 0 for the total acidity The standard error was in the e determinations $< \pm 1$ mN

pH

pH was determined with a Radiometer pH meter type phm 22 p u ing a glass electrode The uncertainty was ± 0 05 pH units

Chloride

0 2 ml of sample was titrated with 20 mN $Hg(NO_3)_2$ with 1 ml of citrate buffer (pH 1 5) and s-diphenylcarbazone (Eastman Kodak 4453) as indicator as according to BRUN (1949) The $Hg(NO_3)_2$ solution was checked daily against a standard solution of 100 mN NaCl The standard error in these determinations was ± 2 mN

MATERIAL

Five men in good physical condition were studied For data about their physical status see Table 1 I No symptoms of gastro intestinal disease and no history of allergy were noted In addition two dogs *with gastric fistulae were used, these are also included in Table 1 I*

METHODS

Procedure

Dog experiments

A fistula was made in each dog by inserting a silver cannula in the lower part of the fundus of the stomach The dogs were not used until four weeks after operation The secretion from the stomach was collected via the cannula into a measuring cylinder which was emptied at periods of about five to ten minutes Before every experiment the dog was fasted for at least twelve hours but was allowed water The animal was stood on a table and restrained in a loose harness Each experiment lasted for about four to five hours The histamine was injected continuously intravenously at a constant rate using a special injection device (see Öbrink, 1948) For concentrations and the volumes of the histamine injected see Table 1 I To avoid side effects of histamine an antihistamine preparation chlorphenirainine (Allergisan® Pharmacia) was injected intravenously before the histamine (cf Kay 1953) The dose of antihistamine was four to twelve mg the *amount being adjusted for the injection rate of histamine*

In order to detect regurgitated material from the duodenum in the samples 0 1 – 0 2 gram of the dye uranine which is secreted in the bile were injected intravenously in a single dose at the same time as the antihistamine

Experiments in man

For purposes of comparison the procedure was as far as possible similar to that in the dog experiments The gastric contents were removed at intervals of about five to ten minutes by suction through a rubber or a plastic tube To prevent the swallowing of saliva this

Sodium and potassium

Sodium and potassium were determined flamephotometrically (Flamespectrophometers used were Perkin Elmer Co. type 52A Series 2, with internal lithium standard or Beckman B). Both sodium and potassium were determined in a concentration range up to 4 mN, the samples being diluted when necessary. A calibration graph was made with standard solutions of KCl and NaCl (range 0.1–4.0 mN). Potassium does not interfere with the determinations of sodium in the presence of concentrations of at least up to 100 mN and the interference of 100 mN sodium on 1 mN potassium is only 5 % (MARSDEN, personal communication). Since the Na concentration when measuring K was never more than about 20 mN the sodium interference was neglected.

CHAPTER 1

The Relation between Injection Rate of Histamine and Gastric Secretion in Man and Dog

It is well established that after a subcutaneous injection of histamine the rate of gastric secretion first rises to a maximum and then declines until secretion finally ceases after about an hour. In studying the relationship between the dose of histamine and rate of gastric secretion this mode of histamine administration is unsatisfactory. It is desirable to obtain steady state conditions, i.e. to maintain a constant stimulus and thus a constant rate of secretion.

TEORELL (1933) pointed out that a constant gastric secretory rate could be obtained by the continuous intravenous injection of histamine. In 1937 he presented the theoretical relationships between the injection rate and the concentrations of histamine in blood and tissue. He suggested that the steady state concentration should be directly proportional to the injection rate.

OBRINK (1948) confirmed the correctness of this hypothesis in studies on the concentration of histamine in plasma after continuous intravenous injection. He found a definite relationship between the injection rate of histamine and rate of gastric secretion for Heidenhain pouch dogs which could be described by the exponential equation

$$s = a(1 - e^{-kr}) \qquad (1.1)$$

where s = the rate of gastric secretion (ml/min)
r = the injection rate of histamine (mg/hour)
and a and k are constants.

Previously only preliminary studies of this relationship have been made in man (NORDGREN 1958).

The purpose of the work described in this chapter was to study this problem in more detail with special reference to three parameters:

1. The maximal rate of gastric secretion
2. The sensitivity of the mucosa to histamine

Sodium and potassium

Sodium and potassium were determined flamephotometrically (Flamespectrophotometers used were Perkin Elmer Co type 52A Series 2 with internal lithium standard or Beckman B) Both sodium and potassium were determined in a concentration range up to 4 mN the samples being diluted when necessary A calibration graph was made with standard solutions of KCl and NaCl (range 0 1–4 0 mN) Potassium does not interfere with the determinations of sodium in the presence of concentrations of at least up to 100 mN and the interference of 100 mN sodium on 1 mN potassium is only 5 % (MARSDEN personal communication) Since the Na concentration when measuring K was never more than about 20 mN the sodium interference was neglected

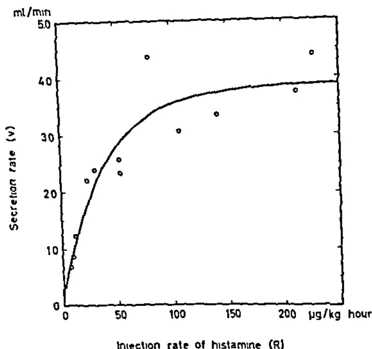


Fig 1 The relationship between injection rate of histamine and rate of gastric secretion during steady state conditions (Dog M)

different steady injection rates of histamine R (µg/kg body weight/hour) and the rates of secretion v (ml/min) from different experiments on the same dog is given in Fig 1 2 Each point in Fig 1 2 represents the mean secretion rate during the steady state in a single experiment (see Fig 1 1) The continuous line was obtained by fitting the experimental values to Eq 1 2 (see below) using the method of least squares

Experiments in man

Fig 1 3 illustrates a typical experiment on a human subject With different injection rates of histamine correspondingly different rates of secretion were obtained In experiments without histamine stimulation there was always a basal secretion The results were expressed in the same way as in the dog experiments (see Fig 1 4)

In order to evaluate the total loss of water during an experiment

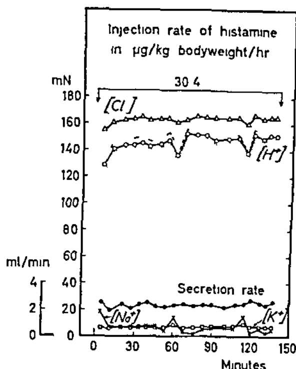


Fig 1 1 A typical experiment with continuous intravenous injection of histamine and a steady rate of gastric secretion with fairly constant [H^+] [K^+] [Na^+] and [Cl^-] The [H^+] was titrated with bromthymolblue (circles) in addition to Topfer s reagent Dashed lines total acidity and dotted lines free acidity (Dog M)

3 The secretion in the absence of histamine stimulation (basal secretion)

In previous work on the human stomach or intact stomach in dog it has generally not been possible to assess the magnitude of admixture contributed by the saliva or duodenal contents In the technique described here special attention has been paid to the presence of the e disturbing factors

Results

Dog experiments

The results of a typical experiment on a dog are shown in Fig 1 1 It can be seen that the rate of secretion and the acidity [H^+] reach a fairly constant level which is maintained throughout the experiment i e a steady state is obtained A graph of the relation between

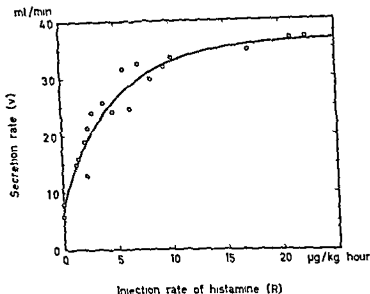


Fig 1 4 The relatio sh p between injection rate of histamine and rate of gastric secretion in man during steady state cond tions (Subject B I)

through the pylorus a steady secretion rate would be unlikely to be recorded As a rule a steady rate was in fact observed

Only those experiments where steady state levels were attained were included in the analysis of the results In contrast to the experi ments on man contamination by large amounts of saliva and duo denal secretion in several dog experiments necessitated their exclusion from the acceptable material

Some general comments on the results

The accuracy with which the gastric secretion rate can be measured is of great importance For this reason special attention has been paid to the problem of contamination by the admixture o saliva or duodenal secretion

Saliva

In addition to the saliva sucker in the mouth (v s) a plastic tube connected to a suction device was in some experiments in man placed in the lower part of the oesophagus (about 40 cm from the teeth)

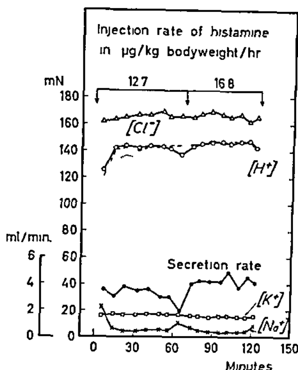


Fig 1 3 A typical experiment in man (Subject K G K) The notation is the same as in Fig 1 1

the total amount of gastric contents and saliva collected from the subjects is shown in Table 1 I where also the volume of saline injected along with the histamine is given The total water loss was not determined in the dogs because it was considered that a salivary suction fixed in the mouth might be irritating There was thus some additional unrecorded water loss due to salivary spilling from the mouth

The juice collected might of course contain an admixture of saliva and duodenal secretion In order to evaluate the extent of this contamination the procedure used in the experiments was found to be satisfactory for the following reason Both the rate of gastric secretion and the acidity were obtained at steady state levels An admixture of the gastric secretion of saliva or duodenal secretion increases the apparent rate of secretion but lowers the acidity (Nordgren 1958) A real increase in secretion rate is never accompanied by a decrease in the acidity and if this happens it must imply an extragastric admixture Such an event is illustrated in Fig 1 5 and it seems valid to exclude such values If there was a sudden loss of gastric contents

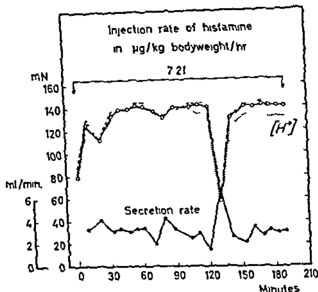


Fig 1 5 An experiment in man demonstrating the occurrence of extragastric admixture (Subject B I)

to remove saliva and possible oesophageal secretion The largest amount collected during a 3 to 4 hours experiment was not more than 3 ml indicating that fluid movement between the mouth oesophagus and the stomach (and the reverse direction) was negli gible It is shown in Table I I that large amounts of saliva were collected through the mouth sucker during each experiment

Duodenal regurgitation

To detect duodenal regurgitation a dye test was used (NORDGREN to be published) A number of different dyes were injected intrave nously in cats during histamine stimulated gastric secretion Of the dyes tested uranine was found most suitable because

1 it gives an extremely intense colour easily seen with the eye and detectable photofluorometrically in concentrations of 1 10^7
2 it is not secreted by the gastric mucosa
3 a considerable amount of the dye passes via the bile to the duo denum and colours vividly all the duodenal contents
4 it has a low toxicity (FUJII and SAKATA 1953)

TABLE 1 I *Data of the material*

Subject	Weight kg	Length cm	Body surface[a] m²	Age in years	Dose of histamine range μg/kg hr	Amount of fluid injected (ml)		Gastric contents withdrawn (ml)		Amount of saliva withdrawn (ml)		
						Mean	Range	Mean	Range	No of experiments	Mean	Range
K G K	77 0	190	2 04	26	3 1–21 1	12 9	9 5–15 5	422 8	259 7–578 5	5	337	220–537
B I	72 0	191	1 99	22	1 3–22 4	13 5	10 0–18 4	449 1	276 2–693 0	17[b]	208	95–420
H C	53 0	179	1 66	17	3 6–22 6	22 4	12 2–30 0	369 3	319 4–513 1	3	258	240–285
R N	51 5	—	—	15	3 7–20 1	16 2	10 0–24 9	506 3	319 2–687 1	5	209	155–370
G L	51 7	171	1 59	16	1 9–27 9	20 4	16 4–26 3	246 2	166 7–419 4	5	276	190–390
Dog I	22 5	—	—	—	7 0–36 3	12 2	5 6–19 1	271 8	161 7–458 5			
Dog M	19 2	—	—	—	6 8–227 3	12 7	9 0–22 5	369 6	127 7–581 8			

[a] Body surface calculated as according to Du Bois and Du Bois (1916)
[b] In 5 experiments the pH of the saliva was measured (mean 7 9 range 7 6–8 3)

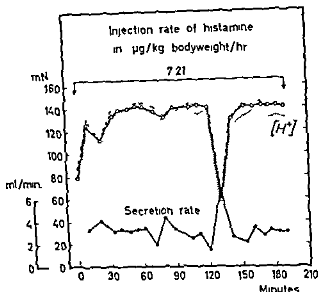


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3 a considerable amount of the dye passes via the bile to the duodenum and colours vividly all the duodenal contents
4 it has a low toxicity (Fujii and Sakata 19.3)

TABLE I II *Secretion rate and acidity before and after administration of chlorpheniramine*

Subject	Dose of Allergisan given mg	Secretion rate: without Allergisan ml/min	Secretion rate: without Allergisan of a_1*	Secretion rate: with Allergisan ml/min	Secretion rate: with Allergisan of a_1*	difference of a_1 after Allergisan	Acidity [H+] without Allergisan mN	Acidity [H+] with Allergisan mN
C (exp 2)	12 0	1 27	37 6	1 37	40 5	+2 9	110	109
L (exp 5)	8 8	1 04	37 7	1 00	36 2	−1 5	96	95
N (exp 4)	12 0	0 31	9 7	0 34	10 6	+0 9	85	88
I (exp 11)	8 0	1 19	32 4	1 14	31 1	−1 3	100	106

*a_1 = maximal rate of gastric secretion

Because of the appearance of some bile in experiment 11 (B I) this was not used when calculating the regression curve between the injection rate of histamine and rate of gastric secretion

The appearance of uranine in the gastric contents was taken as evidence of duodenal regurgitation

The effect of antihistamine on the gastric secretion

As chlorpheniramine was used to obviate the side effects of hista mine it was of importance to exclude any possible effect of the anti histamine on the parameters under investigation Chlorpheniramine (Allergisan®) in a dose of 8 to 14 mg therefore was administrated intravenously in a single dose during a period when there was steady rate of fluid efflux from the stomach induced by continuous histamine injection The secretory rate was kept below 40 % of the maximal theoretical rate of secretion in order to make the subject more sen sitive to the possible effects of the antihistamine drug At this level of gastric secretion changes should exert relatively large effects on the secretory rate (Fig 1 4) As can be seen in Table I II only small changes were obtained after the administration of the chlorphenira mine This is in accordance with many earlier reports (MCELIN and HORTON 1946 SANGSTER GROSSMAN and IVY 1946 MOERSCH 1946 WOOD 1948 RAGINS BENDITT GREENLEE and DRAGSTEDT 1958) but it should also be noted that a slight stimulating effect of the parenterally administered antihistamine has been reported (LINDE 1950)

Discussion

The results indicate a correlation between the rate of gastric secretion and the injection rate of histamine in both man and the dog

For the analysis of the dynamics of gastric secretion it is necessary to evaluate at least three parameters

(i) The maximal secretory capacity (*MSC*) ml/min
(ii) An index of the sensitivity of the gastric mucosa to stimulation
(iii) The basal secretion rate i e the secretion without histamine stimulation ml/min

Procedure for measurement of the parameters

The basal secretion was measured directly but numerical values of parameters (i) and (ii) can only be calculated indirectly as follows

The relation between the injection rate of histamine and the rate of gastric secretion in Heidenhain pouch dogs was found by ÖBRINK (1948) to be described by the equation

$$v = a\,(1 - e^{-qR}) \qquad (1\ 1)$$

where v = the rate of secretion (ml/min) R = the injection rate of histamine (mg/kg body weight hour Öbrink used r) a = the maximal rate of secretion and q = a constant (Öbrink u ed k) The locus of this equation is a line passing through the origin which means that in the absence of histamine stimulation the rate of gastric secretion is zero This is only a special form of the more general case where there is a basal secretion and which is described by the equation

$$v = a_1 - a_2 e^{-qR} \qquad (1\ 2)$$

where v q and R are the same as in Eq 1 1 a_1 = the maximal rate of secretion (ml/min) and a_2 = the maximal increment in the secretion rate obtained with histamine stimulation

In the human subjects there was always a distinct basal secretion (v i) and therefore Eq 1 2 has been used throughout this paper No attempts were made to fit the experimental data to other types of equations as Eq 1 2 seemed satisfactory in most cases [1]

[1] In calculating the regression line the total sum of squares is defined as

$$\sum d_t^2 = \sum (v_i - v)^2$$

where v_i the ith observation of the rate of the gastric secretion v = the

TABLE 1 II *Secretion rate and acidity before and after administration of chlorpheniramine*

Subject	Dose of Allergisan given mg	Secretion rate: without Allergisan ml/min	of a_1*	with Allergisan ml/min	of a_1*	difference of a_1 after Allergisan	Acidity [H⁺]: without Allergisan mN	with Allergisan mN
H C (exp 2)	12.0	1.27	37.6	1.37	40.5	+2.9	110	109
G L (exp 5)	8.8	1.04	37.7	1.00	36.2	−1.5	96	95
R N (exp 4)	12.0	0.31	9.7	0.34	10.6	+0.9	85	88
B I (exp 11)	8.0	1.19	32.4	1.14	31.1	−1.3	100	106

*a_1 = maximal rate of gastric secretion

Because of the appearance of some bile in experiment II (B I) this was not used when calculating the regression curve between the injection rate of histamine and rate of gastric secretion

The appearance of uranine in the gastric contents was taken as *evidence of duodenal regurgitation*

The effect of antihistamine on the gastric secretion

As chlorpheniramine was used to obviate the side effects of histamine it was of importance to exclude any possible effect of the antihistamine on the parameters under investigation Chlorpheniramine (Allergisan®) in a dose of 8 to 14 mg therefore was administrated intravenously in a single dose during a period when there was steady rate of fluid efflux from the stomach induced by continuous histamine injection The secretory rate was kept below 40 % of the maximal *theoretical rate of secretion in order to make the subject more sensitive* to the possible effects of the antihistamine drug At this level of gastric secretion changes should exert relatively large effects on the secretory rate (Fig 1 4) As can be seen in Table 1 II only small changes were obtained after the administration of the chlorpheniramine This is in accordance with many earlier reports (MCELIN and HORTON 1946 SANGSTER GROSSMAN and IVY 1946 MOERSCH 1946 WOOD 1948 RAGINS BENDITT GREENLEE and DRAGSTEDT 19..) but it should also be noted that a slight stimulating effect of the parenterally administered antihistamine has been reported (LINDE 1950)

TABLE 1 III The degree of fit with Eq 1 2

Subject	No of experiments	Σd_1^2	Σd^2	$100\left(1-\frac{\Sigma d_1^2}{\Sigma d_1}\right)\%$
K G K	11	0.0[illegible]	17.18	95.9
B I	17	0.986	9.679	89.8
H C	7	0.46	[illegible].9[illegible]4	84.2
R N	5	0.637	1.117	43.0
G L	9	0.169	3.552	95.[illegible]
Dog L	8	3.391	8.611	60.6
Dog M	13	[illegible].431	18.349	88.5

The product vR has been plotted against R in Fig 1 6 Eq 1 3 has an inflexion point (i e the second derivative = 0) at an R value of $2/q$ Beyond the point of inflexion the curve rapidly tends to linearity (cf Fig 1 6)

The slope of the tangent at the point of inflexion is

$$\tan\alpha = f'(2/q) = a_1 + a_2/e^2 \qquad (1\ 4)$$

The full equation of the tangent will consequently be

$$vR - f(2/q) - f'(2/q)\,(R - 2/q) = 0 \qquad (1\ 5)$$

This gives an intersection on the R axis

$$R_{R\ 0} = \frac{4a}{q\,(a_1 e^2 - a_2)} \qquad (1\ 6)$$

The slope ($\tan\beta$) of Eq 1 3 in the origin ($f'(R)$ for $R = 0$) is

$$\tan\beta = a_1 - a_2 \qquad (1\ 7)$$

This slope was however difficult to evaluate graphically (see Fig 1 6) and was therefore not used But it should be remembered that $a_1 - a_2$ equals the basal secretion (b) that can be determined experimentally Thus

$$a_2 = a_1 - b \qquad (1\ 8)$$

If Eq 1 8 is substituted in Eq 1 4 and $\tan\alpha$ determined graphically a_1 can be calculated Knowing a_1 a_2 can be calculated from Eq 1 8

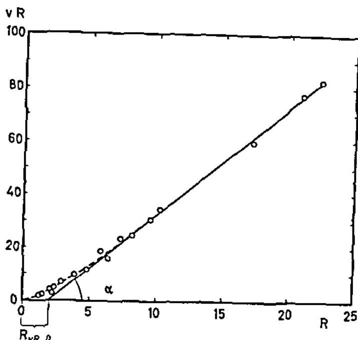


Fig 1 6 The product ιR plotted against ι The dotted line was obtained from Eq 1 3 and the continuous line corresponds to Eq 1 5 The slope of the continuous line is tan α and its intercept on the x axis corresponds to $R_{\iota R=0}$ (Subject B I)

Discussion of an "approximation" test of the parameters

To determine the regression curve of Eq 1 2 a considerable amount of experimental data is required this introduces difficulties into its use in human physiology or clinical medicine It might however be possible to characterize the stimulation–effect relationship on a basis of Eq 1 2 by a more rapid estimation of the parameters in the following way If both sides of Eq 1 2 are multiplied by R we obtain

$$\iota R = R\,(a_1 - a_2 e^{-qR}) \qquad (1\ 3)$$

$$\text{or } \iota R = f(R)$$

---

mean of all ι_i s The residual sum of squares is defined as the sum of the squared deviations of the experimental points from the locus of Eq 1 2 in which the parameters have been estimated according to the I S method $1 - \sum d_i^2/\sum d^2$ (where $\sum d_i^2$ is the sum of the least square distances of the experimental points from the regression line Eq 1 2) can be used as reasonable measure of the degree of fit of the experimental points of the regression curve in the individual subjects (cf for instance SNEDECOR 1956) The expression $(1 - \sum d_i/\sum d^2)$ may however be somewhat influenced by the numerical value of a_1

Table 1 III shows that values of $100\,(1 - \sum d_i/\sum d_2)$ up to 96 were obtained An analysis of the entire data using Eq 1 2 is given in Table 1 IV

TABLE 1 III *The degree of fit with Eq 1 2*

Subject	No of experiments	Σd_1^2	Σd_2^2	$100\left(1-\frac{\Sigma d_1^2}{\Sigma d}\right)$
K G K	11	0 05	17 182	95 9
B I	17	0 986	9 679	89 8
H C	7	0 467	2 974	84 2
R N	5	0 637	1 117	43 0
G L	9	0 169	3 57	95 [illegible]
Dog L	8	3 391	8 611	60 6
Dog M	13	491	18 349	86 5

The product vR has been plotted against R in Fig 1 6 Eq 1 3 has an inflexion point (i e the second derivative $=0$) at an R value of $2/q$ Beyond the point of inflexion the curve rapidly tends to linearity (cf Fig 1 6)

The slope of the tangent at the point of inflexion is

$$\tan \alpha = f' (2/q) = a_1 + a_2/e^2 \tag{1 4}$$

The full equation of the tangent will consequently be

$$vR - f(2/q) - f' (2/q) (R - 2/q) = 0 \tag{1 5}$$

This gives an intersection on the R axis

$$R_{R\ 0} = \frac{4a}{q(a_1 e^2 - a_2)} \tag{1 6}$$

The slope ($\tan \beta$) of Eq 1 3 in the origin ($f' (R)$ for $R = 0$) is

$$\tan \beta = a_1 - a_2 \tag{1 7}$$

This slope was however difficult to evaluate graphically (see Fig 1 6) and was therefore not used But it should be remembered that $a_1 - a_2$ equals the basal secretion (b) that can be determined experimentally Thus

$$a_2 = a_1 - b \tag{1 8}$$

If Eq 1 8 is substituted in Eq 1 4 and $\tan \alpha$ determined graphically a_1 can be calculated Knowing a_1 a_2 can be calculated from Eq 1 8

$R_{vR=0}$ in Eq 1 6 can be determined graphically and in Eq 1 6 q is now the only unknown parameter and can thus be evaluated

The difficulty seemed to be to determine the slope of the tangent at the point of inflexion, but scrutiny of Fig 1 6 shows that the bend of the curve at this point is so slight that the slope will be approximately the same even if it is not drawn through the point of inflexion

A test was made to see how far from the point of inflexion in Eq 1 3 the tangent could be drawn using the range of the R values employed in the human subjects with varying q values Fixed a_1 and a_2 values (the mean of the calculated a_1 and a_2 respectively) were used with varying q values inserted in Eq 1 3 A low q value gives the point of inflexion $(2/q)$ an R value far greater than the experimental R values It can be shown that for q values below 0 05 the graphical method gives an overestimate Thus the construction of a tangent here could involve a rather uncertain extrapolation of the curve calculated from Eq 1 3

It is of interest to consider the information which can be elicited if for instance only two experimental points as for example in a simple clinical test are available To examine this matter the positions of *the tangents and R axis intercepts ($R_{iR=0}$) drawn from any two* points of a whole set of experimental points from the same individuals were estimated The minimal distance between any pair of points used was *4 5 R* units and only *points lying on or about the graph* in its straight part were used A typical set of such points is shown in Fig 1 6 The entire material was treated in this way The results show in the main reasonable accord *(see Table 1 V) although some* rather wide variations occur in the latter cases however the degree of fit $(1-\Sigma d_1/\Sigma d^2$ in Table 1 III) was also rather poor Obviously the more experimental points that can be determined *the more accurate* will be the values of $\tan \alpha$ and $R_{vR=0}$

The basal secretion rates were not included when the parameters in Eq 1 3 were determined by *the method of the least squares For* comparison the parameters were included in one subject (B I) including the basal secretion in Eq 1 3 giving $q=0\ 20$ $a_1=3\ 72$ $a_2=2\ 98$ S (the standard error of estimate)=*0 26 compared with* $a_1=3\ 67$ $a_2=2\ 79$ $S=0\ 26$ (with the basal secretion excluded)

TABLE 1 IV *The values of the parameters determined*

S bj ct	No of observa tions	Basal secre t on mean	Calculat ons with the method of the lea t squares				a_1 per kg body weight	a_1 per m² body surface	Basal secretion in % of a_1	FD_0 / g/kg hr of hi tamine	Calculation with the graphical method	
			a_1 ml/min	a_2 ml/min kg	q hr/μg	S					a_1 ml/min kg	q hr/μg
K G K	11	0 50	6 90	6 34	0 05	0 30	0 0896	3 38	8 6	13 8	.47	0 09
B I	17	0 0?	3 67	79	0 ?0	0 ?7	0 0510	1 84	16 9	3 5	3 6	0
H C	7	0 ?8	3 38	? ?0	0 08	0 34	0 0638	-.04	8 3	0 2	3 11	0 11
R N	5	0 75	3 0	1 51	0 15	0 6	0 00?1	—	23 4	4 7	3 06	0 17
G L	9	0 6	2 76	? 55	0 08	0 17	0 0534	1 74	0 4	10 0	-.39	0 10
Dog L	8	0 86	3 8	4 4	0 07	0 8	0 1698	—	5	15 0	4 50	0 44
Dog M	13	—	3 86	3 70	0 03	0 50	0 ?010	—	—	8 0	—	—

Discussion of the various parameters

The basal secretion

In Heidenhain pouch dogs no basal secretion was found (Öbrink 1948) In the present experiments in dogs with intact stomachs a basal secretion was not conclusively demonstrated In man, however the basal secretion does not cease even after extended fasting Dragstedt Oberhelman Jr and Woodward (1951) suggested that the basal secretion was under central nervous control as it was markedly reduced after vagotomy

In the present material there was considerable variation of the basal secretion rate in the same subject between different experiments The mean values for each subject varied between 8 and 23 (mean 14) per cent of their corresponding *MSC* values On account of the low acid output the acidity necessary to keep the pylorus closed may not always have been maintained Some acid may therefore have been lost through the pylorus which could possibly explain some of the observed variations in the basal secretion rate

The maximal secretion rate (a_1)

Several attempts to determine *MSC* with intravenous infusion of histamine have been reported Öbrink (1948) Linde (1950) and Nordgren (1958) used Eq 1 1 to calculate the *MSC* Adams *et al* (1954) calculated the *MSC* from a logistic curve after subtracting the basal secretion Hansson *et al* (1948) and Wood (1948) considered they had reached the *MSC* when a bigger dose of histamine gave no significant rise in output from the stomach Table 1 VI gives a survey of the estimated values obtained from steady state conditions after histamine injection

Even if it has not been proved rigorously that the acid originates from the parietal cells there is much indirect supporting evidence Thus Card and Marks (1957) in man and Marks Komarov and Shay (1958 1960) in dog reported a correlation between acid output and parietal cell mass In patients with atrophic gastritis Guiss and Stewart (1948) found a relationship between the acid output and the estimated number of parietal cells left intact Card and Marks (1960) in man also reported a correlation between the number of parietal cells in the excised part of the stomach and a diminished acid output The frequency of parietal cells per unit area of gastric

Table 1 V *The variation of* $\tan \alpha$ *and* $R_{s=0}$ *(Fig 1 5) when only two points along the slope are used to estimate them*

Subject	The whole material used		Only two points used	
	$\tan \alpha$	$R_{s=0}$	$\tan \alpha$	$R_{s=0}$
K G K	6 14	5 15	5 65	4 05
			5 92	4 80
			6 05	5 00
			6.35	5 90
			6 50	6 30
			6 0	6 10
			7.30	7 00
B I	4 03	1 90	3 95	1.50
			4 00	1 60
			4 08	2 10
			4 08	2 10
			4 10	2 0
			4 10	2 90
H C	3 0	4 10	3 8	3 0
			4 00	4 30
R S	3.38	0	2 95	1 40
			3.38	2.20
G L.	68	4 0	2 46	3 0
			2 5	2 90
			2 64	4.30
			0	4 40
			2 4	4 90
			2 9	5 00
Dog L	06	9 0	3 40	−0 30
			4 47	8 40
			5 60	8 00
			6 17	10 00
Dog M	4 47	9 50	4 40	3 00
			4 55	37 00
			5.55	47 0
			6 10	63.0

mucosa was correlated to the acid output according to TONGEN (1950) Variations in the number of parietal cells may therefore be expected to be reflected by differences in the *MSC*

Attempts to correlate *MSC* and weight or age of the subject were

Discussion of the various parameters

The basal secretion

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tion (relative to the MSC) A suitable measure of the sensitivity would therefore seem to be the injection rate of histamine evoking 50% of the maximal rate of secretion This injection rate might be called the Effective Dose 50 (ED_{50}) (see Table 1 IV) ED_{50} can be determined if the parameters a_1 and a of Eq 1 2 are known and $ED_{50} = 0\,693/q$, i e ED_{50} is in fact independent of a_1 and a_2 It can also be determined from the sigmoid relation between the dosage of histamine (log R) and the rate of secretion

A high numerical value of q (i e a low value of the ED_{50}) indicates a high sensitivity of the gastric mucosa to histamine The term *hyper secretion* a word often used in a rather vague way might therefore be defined more quantitatively in terms of a high q (or a low ED_{50}) value or a high MSC value or a combination of both Hypersecretion would thus refer to the sensitivity of the response of the gastric mucosa to a submaximal (e g 50%) histamine stimulus as well as to the magnitude of the maximal secretory rate which is correlated with the number of parietal cells

TABLE I VI *Previous determinations of the maximal secretory capacity (MSC) in the whole stomach*

Authors	Species	No of subjects	Type of stomach preparation	Maximal secretory capacity (MSC)
Hansson Grossman Ivy (1948)	dog	6	total stomach vagally denervated	0 67–2 70 ml/min
Hansson Grossman Ivy (1948)	dog	4	intact stomach	1 15–1 70 *ml/min*
Hansson Grossman Ivy (1948)	man	1	intact stomach	4 75 ml/min (submax capacity)
Nordgren (1958)	man	11	intact stomach	1 56–4 64 ml/min
Wood (1948)	cat	6	intact stomach	8 42 ml/hr

not successful (NORDGREN 1958) nor could COX (1952) find any correlation between the weight or the surface area of the stomach and *the body weight or the age*

Table I IV shows the quotient between a_1 (the *MSC*) and the body weight or body surface The body weight quotient was definitely bigger in the dogs than in man which may be a reflection of a bigger parietal cell mass per unit body weight in the dogs In the present investigation the relation between *MSC* (a_1) and the body weight was found relatively constant and may therefore possibly be a rough measure of the magnitude of this quot*ient in a population* of healthy subjects

The sensitivity index q

Measurements of the sensitivity of response of the gastric mucosa to stimulation are few (ÖBRINK 1948 HUNT and KAY 1954 NORDGREN 1958) The *MSC* should be a measure of the number of parietal cells However it gives no information of the *sensitivity of the* parietal cells to histamine stimulation although this might be of equal or even greater importance than the *MSC* itself

In this study a bigger q value means that a lower injection rate of histamine suffices to produce the same relative rate of gastric secre

through the mucosa (i e back diffusion of H^+ from the stomach to the circulation) causes the changes in the concentrations of the electrolytes observed at different secretion rates

"The primary acidity"

The primary acidity C_0 (i e the acidity which the parietal cell is postulated to secrete) was studied through instillation of a buffer glycine into the stomach thus reducing backdiffusion of H^+ from the stomach In cats TEORELL (1940) calculated C_0 to be 208 mN and in dogs LINDE TEORELL and ÖBRINK (1947) calculated the C_0 value to be 169 mN rising to 464 mN with diminishing rate of gastric secretion Their highest analytical value was 354 mN In man IHRE (1938) got a value of 143–145 mN and KAIJSER (1943) a maximum of 155 mN when determining C_0 with glycine instillations On account of difficulties in avoiding extragastric admixture and losses of glycine the results in man must be considered as somewhat uncertain

The *necessity of constant secretion rates* when analysing the present relationship should be stressed In non steady states time lags obscure the relations cf ÖBRINK s lake hypothesis (1948)

The relationship has been thoroughly investigated only for gastric pouches and there is a dearth of information from *intact stomachs* The purpose of this investigation was therefore to study the relationship between the rate of gastric secretion and the acidity in man and dog Continuous intravenous injection of histamine was used to get steady state conditions necessary for quantitative analysis of the relationship

Results

Dog experiments

In Fig 1 1 a typical experiment is shown When the rate of secretion is constant the acidity also becomes constant The relatively small amounts of bound acid determined through titration with Topfer s reagent indicate that there are practically no weak acids or buffering substances in the secretion In Fig 2 1 the steady state acidity levels are plotted against the corresponding rates of secretion (r) obtained in every such experiment The regression curves were

CHAPTER 2

The Relation between the Rate of Gastric Secretion and the Acidity during Steady State Conditions in the Intact Stomach of Man and Dog

The existence of a simple relationship between the rate of gastric secretion and acidity has been a matter of much discussion HOLLANDER and GOWGILL (1931) found that when a single massive dose of histamine was given subcutaneously the acidity rose and remained at a constant level for a rather long time while at the same time the secretion rate reached a peak and then declined again This seemed to contradict the existence of a simple relationship but GRAY BUCHER and HARMAN (1940) who injected histamine subcutaneously every 10 minutes into dogs obtained a constant rate of gastric secretion and suggested there was a hyperbolic relationship between the rate of gastric secretion and the acidity

A prerequisite for proving a fixed relationship seems to be a constant secretion rate

ÖBRINK (1948) in Heidenhain pouch dogs and NORDGREN (1958) in man studied the rate of secretion and the acidity with continuous intravenous injection of histamine ÖBRINK (1948) found that the following relation fitted well with the experimental data

$$H = \frac{C_0}{\frac{k}{v} + 1} \qquad (2\ 1)$$

where H = the observed acidity (mN)

C_0 = the primary acidity (mN)

v = the secretion rate (ml/min) and

k = a permeability coefficient (ml/min)

Eq 2 1 was deduced by TEORELL (1947) from his ' diffusion theory which thus found experimental support In the diffusion theory he suggested that the diffusion of hydrogen ions and other electrolytes

through the mucosa (i e back diffusion of H^+ from the stomach to the circulation) causes the changes in the concentrations of the electrolytes observed at different secretion rates

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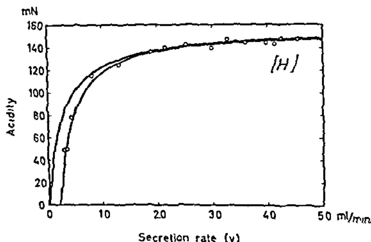


Fig 2 2 The relationship between rate of gastric secretion and acidity in man (Subject K G K) Further see legend of Fig 1

probably of less significance than that of extragastric admixture An example of this is seen in Fig 2 2 where of the two points with the lowest acidity one is obtained with and the other without injection of an antisecretory drug

Experiments in man

Fig 1 3 illustrates a typical experiment As in the dogs only a small amount of bound acid was present In Fig 2 2 the steady state values of the acidity from the same subject (K G K) are plotted against the corresponding gastric secretion rates

Discussion

The quantitative relationship between rate of gastric secretion and acidity in man and dog with intact stomach was determined

Correlation to the theoretical relation used

In Figs 2 1 and 2 2 it can be seen that the experimental points fit rather well with the regression curves The left curve in the figures

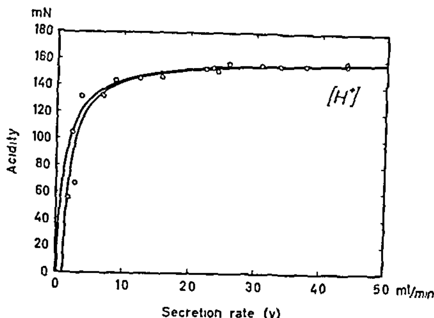


Fig 2 1 The relationship between rate of gastric secretion and acidity (titrated with bromthymolblue) during steady state conditions The left curve in the figure was obtained from Eq 2 1 and the right curve from Eq 2 2 (Dog M)

obtained by using the method of the least squares on Eq 2 1 (the left curve) and on Eq 2 2 on the right curve respectively (see below)

Reduction of extragastric admixture with drugs

In both man and dog in experiments with low rates of gastric *secretion* extragastric admixture was sometimes a serious source of error and thus a steady gastric secretion rate could not always be obtained In order to diminish the possibilities of extragastric admixture *in the gastric secretion by the saliva bile and pancreatic juice* the following inhibitory drugs were given in some experiments Propantelin (Probanthine® Searle) total dose 6 0–13 5 mg or Dixamon (*Banthine® Searle) total do e 20 0–32 5 mg They were given as* single intravenous doses during the first part of the experiments

These inhibitory drugs also to a certain extent inhibited the histamine *stimulated gastric secretion so that a lower rate of gastric secretion* was obtained compared to when the drugs were not administrated The relationship *between* acidity and secretion rate during steady *state might possibly* be somewhat disturbed in the same way as NORDGREN and ÖBRINK (1956) showed that atropine caused an increased acidity for a *fixed* secretion rate but such a *disturbance* was

TABLE 2 I *Data and calculated parameters of the acidity–secretion rate relationship*

Subject	Number of experiments	Calculations with the method of the least squares: $H = C\left(1 - \frac{k}{v}\right)$ C mN	k ml/min	s	$H = \frac{C}{\frac{k}{v}+1}$ C mN	k ml/min	s	Graphical calculation C mN	k ml/min	[H] at v = 5	Highest [H] measured at the titration mN
K G K	15	153 4	0 2	9 0	158 0	0 35	11	15 5	0	139 5	149 0
B I	18	154 3	0 31	7 9	157 5	0 4	11 7	154 0	0 30	135 0	145 0
H C	10	140 8	0 10	9 3	153 1	0 43	7 6	150 0	0 33	130 0	139 0
R N	1	1 63	0 24	17 0	130 3	0 38	0 5	140 0	0 51	114 0	1 9 0
G L	13	138 0	0 3	9	153 4	0 52	5 4	148 0	0 34	1 7 0	1 7 0
Dog L	8	16 1	0 5	1 4	161 0	0 6	24 0	166 0	0 34	146 0	16 0
Dog M	17	160 9	0 11	3 5	16	0 14	5 9	161 5	0 15	154 0	161 0

was obtained when using the method of the least squares on Eq 2 1 Eq 2 1 postulates the complete and immediate mixing of the acid secreted With this condition TEORELL (1947) regarded the concentration gradient between the gastric acid secreted and the blood as $[H^+]-0$ If however mixing is incomplete the concentration gradient will be maximally C_0-0 instead of $[H^+]-0$ ÖBRINK (1948) treated this case and derived the equation

$$H=C_0\left(1-\frac{k}{t}\right) \qquad (2\,2)$$

The symbols are the same as in Eq 2 1

As can be seen from Figs 2 1 and 2 2 Eq 2 2 seems to fit slightly better than Eq 2 1 indicating the existence of some degree of incomplete mixing of the acid secreted

In Table 2 I the primary acidity (C_0) and the permeability coefficient (k) were determined for the whole material using both Eqs 2 1 and 2 2 The sum of the least squares was used to indicate which equation fitted best

Graphical determination of the primary acidity and the permeability coefficient

For the graphical determination of C_0 and k which is a quicker procedure the product tH (i e the acid output per minute) was plotted against t (Fig 2 3) cf ÖBRINK (1948) and HEINZ and ÖBRINK (1954) The dotted curve in Fig 2 3 was derived from Eq 2 1 and can be written

$$tH=tC_0-kH \qquad (2\,3)$$

(with the same symbols as in Eq 2 1)

Similarly Eq 2 2 gives (both sides multiplied by t and rearranged)

$$tH=tC_0-kC_0 \qquad (2\,4)$$

This is the straight line of Fig 2 3 (C_0 and k are assumed to be constants)

For high values of t Eq 2 3 and Eq 2 4 will be almost identical because

$$kH\to kC_0 \qquad (2\,5)$$

when $t\to\infty$

TABLE 2 1 *Data and calculated parameters of the acidity–secretion rate relationship*

Subject	Number of experiments	Calculati n with the m th od of th e lea t s q ares						Graphical calculation		[H] at $v = 5$	H µ] at [H⁺] measured at the titration mN
		$H = C\left(1 - \frac{k}{v}\right)$			$H = \frac{C}{\frac{k}{v}+1}$						
		C mN	k ml/min	S	C mN	k ml/min	S	C mN	k ml/min		
K G K	15	153 4	0 [illegible]	0 0	158 0	0 36	11 [illegible]	15 5	0	139 5	140 0
B I	18	154 3	0 31	7 0	1[illegible]7 5	0 4	11 7	154 0	0 30	135 0	145 0
H C	10	140 8	0 10	9 3	153 1	0 43	7 6	150 0	0 33	130 0	130 0
B [illegible]	1[illegible]	1 63	0 4	17 0	130 3	0 38	0 5	140 0	0 51	114 0	1 90
G L	13	138 0	0 3	9	153 4	0 5[illegible]	5 4	148 0	0 34	1 70	1 70
D g I	8	16 1	0 5	21 4	161 0	0 6	[illegible]4 0	166 0	0 34	146 0	16 0
D g M	17	160 0	0 11	3 5	16[illegible]	0 14	5 0	161 5	0 15	154 0	161 0

was *obtained when using the method of the least squares on Eq 2 1* Eq 2 1 postulates the complete and immediate mixing of the acid secreted With this condition TEORELL (1947) regarded the concentration gradient between the gastric acid secreted and the blood as $[H^+]-0$ If, however mixing is incomplete the concentration gradient will be maximally C_0-0 instead of $[H^+]-0$ ÖBRINK (1948) treated this case and derived the equation

$$H = C_0 \left(1 - \frac{\lambda}{\iota}\right) \qquad (2\ 2)$$

The symbols are the same as in Eq 2 1

As can be seen from Figs 2 1 and 2 2 Eq 2 2 seems to fit slightly better than Eq 2 1 indicating the existence of some degree of incomplete mixing of the acid secreted

In Table 2 I the primary acidity (C_0) and the permeability coefficient (λ) were determined for the whole material using both Eqs 2 1 and 2 2 The sum of the least squares was used to indicate which equation fitted best

Graphical determination of the primary acidity and the permeability coefficient

For the graphical determination of C_0 and λ which is a quicker procedure the product ιH (i e the acid output per minute) was plotted against v (Fig 2 3) cf ÖBRINK (1948) and HEINZ and ÖBRINK (1954) The dotted curve in Fig 2 3 was derived from Eq 2 1 and can be written

$$\iota H = \iota C_0 - \lambda H \qquad (2\ 3)$$

(with the same symbols as in Eq 2 1)

Similarly Eq 2 2 gives (both sides multiplied by ι and rearranged)

$$\iota H = \iota C_0 - \lambda C_0 \qquad (2\ 4)$$

This is the straight line of Fig 2 3 (C_0 and λ are assumed to be constants)

For high values of ι Eq 2 3 and Eq 2 4 will be almost identical because

$$\lambda H = \lambda C_0 \qquad (2\ 5)$$

when $\iota \to \infty$

TABLE 2 II *The primary acidity C_0 and the permeability coefficient k calculated from the approximation test proposed The meaning of Range v and Range rH is described in the text*

Subject	C_0 mN	k ml/min	Range v	Range rH
K G K	15	0 21	1 89 4 6	256-630
	154	0 25		
	157	0 25		
	157	0 27		
B I	154	0 31	1 50-3 68	181-512
	151	0 4		
	153	0 30		
	149	0 23		
H C	155	0 41	1 27-2 52	150-330
	149	0 30		
	160	0 44		
	15	0 3		
R N	135	0 53	1 72- 49	256-314
	1 6	0 35		
	150	0 6		
	139	0 43		
G L	163	0 47	1 08- 15	98- 8
	156	0 39		
	150	0 42		
	143	0 33		
Dog L	1 3	0.39	1 9 -3 40	84-494
	164	0 18		
	148	0 15		
	133	−0 23		
	16	0 9		
	146	0 0		
Dog M	163	0 16	1 55-4 3	-683
	163	0 13		
	160	0 13		
	1 8	0 10		

and maximum distances (range) between the pairs of points on the x axis (v) and the y axis (rH) With few exceptions the values in Table 2 II agree rather well with the corresponding values in Table 2 I

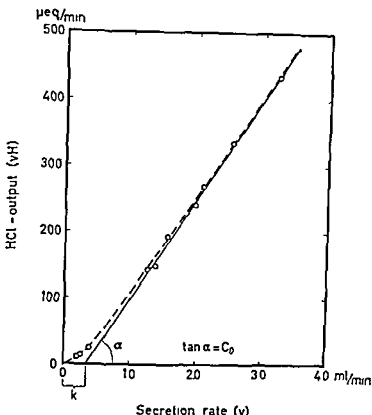


Fig 2 3 Rate of gastric secretion plotted against acid output per unit time The dotted curve represents Eq 2 3 and the continuous line Fq 2 4 Tan α in the figure then equals C_0 *and the x axis intercept of the Fq 2 4 is equal to the* permeability coefficient k (Subject H C)

When applying this graphical method the experimental data always lay along a straight line if the v values were above 1 ml/min It would seem valid to regard the straight part of Eq 2 3 in Fig 2 3 as an approximation to Eq 2 4 The slope from the straight line would represent C_0 *and the intercept of the line on the x axis would be* k Table 2 I shows C_0 and k calculated graphically in this way

Use of an "approximation" test for determination of C_0 and k

In order to determine whether it is possible to evaluate C_0 and k approximately from only two experimental points pairs of points one *in the highest secretion range* and the other at a secretion rate just above 1 ml/min were used (Table 2 II) this table gives the minimum

TABLE 2 II *The primary acidity C_p and the permeability coefficient k calculated from the approximation test proposed The meaning of Range v and Range tH is described in the text*

Subject	C_p mN	k ml/min	Range v	Range tH
K G K	15[illegible]	0 1	1 89-4 6	256-630
	154	0 25		
	157	0 25		
	157	0 7		
B I	154	0 31	1 50-3 68	181-512
	151	0 24		
	1 3	0 30		
	149	0 23		
H C	165	0 41	1 27-2 59	1[illegible]0-330
	149	0 30		
	160	0 44		
	15	0 3		
R N	135	0 53	1 7 2 49	2[illegible]6-314
	1 6	0 35		
	150	0 6[illegible]		
	139	0 43		
G L	163	0 47	1 08-2 1[illegible]	98- 58
	156	0 39		
	150	0 4		
	143	0 33		
Dog L	173	0 39	1 9 -3 40	284-4[illegible]4
	164	0 18		
	148	0 15		
	133	−0 3		
	16	0 [illegible]9		
	146	0 0[illegible]		
Dog M	163	0 16	1 55-4 37	2[illegible]-683
	163	0 13		
	160	0 13		
	158	0 10		

and maximum distances (range) between the pairs of points on the x axis (v) and the y axis (tH) With few exceptions the values in Table 2 II agree rather well with the corresponding values in Table 2 I

The primary acidity

From Table 2 1 it can be seen that in man the primary acidity C_0 is lower than in dogs Before accepting this as true all possibilities that it is an artifact resulting from the experimental or other conditions must be ruled out

The parietal cells are assumed to be the acid secreting cells They are found over the greater part of the body of the intact stomach but the pyloric part (here estimated to contain maximally 20% of the total surface) lacks them (BERGER, 1934 COX Jr 1952) and has no acid secretion This means that in the body of the stomach both secretion and back diffusion of H^+ ions take place while in the pyloric part only back diffusion is possible With incomplete intragastric mixing the amount of acid stored in the pyloric part and the time for this storage would be important In the dog experiments the acid gastric contents were emptied continuously while in man the gastric juice remained in the stomach for some minutes

TEORELL (1939) instilled HCl into the resting stomach of cats and measured the rate at which H^+ ions disappeared As there was no acid being secreted into the stomach the relationship

$$H = H_0 e^{-kt/p} \qquad (2\ 6)$$

described the results well which fits the concept of diffusion with a concentration gradient $[H^+]-0$ between gastric juice and blood

H = the concentration at time t
H_0 = initial concentration at the beginning ($t = 0$) of the instillation
k = the permeability coefficient (ml/min)
p = the volume instilled (ml) and
t = time (min)

The juice secreted into the human stomach remains there until the next emptying During this time it should be expected that in the pyloric part the acidity will be reduced as according to Eq 2 6 The final acidity after such an instillation can be estimated as follows At the highest secretion rates the acidity will be approximately C_0 Applying Eq 2 6 H_0 should thus be equal to C_0 In this discussion we assume both dog and man to have the same value of C_0 (162 mN)

The stomach was emptied at intervals not longer than about ten

minutes and it is assumed that the juice collected was secreted continuously during this period This makes it possible to estimate roughly the values of p and t in the following way

The volume secreted during the first minute has on an average remained in the stomach for 9½ minutes while the volume secreted during the tenth minute has remained for a half minute The whole volume secreted during 10 minutes can be considered to have been instilled during approximately 5 minutes This whole volume constitutes the p and will here be 10 a_1 ml (cf Eq 1 2) The permeability coefficient k was shown in cats (KAIJSER 1943) not to differ significantly between the equally divided upper and lower parts of the stomach 20 % of the surface of the stomach mucosa (the pyloric part) gives $k/5$ The mean k values from Eq 2 1 and Eq 2 2 were determined for each man (see Table 2 I)

The values for H_0 (C_0 mN) p (10a_1 ml) t (5 min) and k can now be inserted in Eq 2 6

The resulting [H+] values were 159 4–160 9 mN showing only a very small reduction of the acidity

Thus the lower C_0 values in man Table 2 I (compared with C_0 in the dogs) are not explained by instillation in the pyloric part As at least some of the gastric secretion in the dog experiments must be expected to flow over the pyloric part (where back diffusion should also take place) these calculated instillations in man would scarcely give even these theoretical differences due to the experimental conditions Consequently *the C_0-differences between man and dog have to be taken for the present as experimental realities*

Comparison of k values for secretion and instillation experiments

As the role of the back diffusion of H^+ ions from an acid secreting stomach back to the circulation has been a matter of much discussion a series of experiments with *intragastric instillation* of HCl were made to determine if the back diffusion (expressed by the permeability coefficient k) could account for the decrease in the acidity after the instillation In one subject (B I) *instillations* of about 30–40 ml of HCl (154–119 mN) were made In each case the stomach was emptied as completely as possible ten minutes after the introduction of the

acid. To check that there was no fluid loss into the duodenum phenol red (estimated by the absorbance at the isobestic point 465 mμ) was added to the instilled solution. PENNER, HOLLANDER and SALTZMAN (1938), HOLLANDER and PENNER (1940), PENNER, HOLLANDER and POST (1940) and ÖBRINK (1948) have used phenolred for this purpose.

The calculation of k from Eq. 2.6 in man is, however, complicated by the basal secretion which is always present. In a resting stomach the acidity of instilled HCl always approaches zero, whereas in a secreting stomach the final acidity will be determined by the secretion rate. ÖBRINK (1963) calculated the time course of the acidity in such an instillation experiment as

$$H = \frac{C_0}{1 + \frac{k}{i}} \left(1 - \frac{1 - \frac{H_0}{C_0}\left(1 + \frac{k}{i}\right)}{\left(1 + \frac{it}{p}\right)^{(1 + \frac{k}{i})}} \right) \tag{2.8}$$

As the values of C_0 and i (basal secretion) were known, k could be solved from Eq. 2.8. Most experiments had to be excluded because of extragastric dilution and loss of acid into the duodenum, but from five experiments a mean value of $k = 0.43$ was obtained.

The mean k value obtained from *secretion experiments* without instillation of acid was 0.36 (from both Eqs. 2.1 and 2.2). Both values are certainly liable to errors due to experimental uncertainties. They are, however, of the same order which seems to support the view that the acidity decrease can be explained on a basis of back diffusion.

Introduction of glycine to determine C_0

To estimate the primary acidity (C_0) at secretion rates other than the maximal ones a glycine buffer (0.25–0.33 N) was used as according to TEORELL (1940). Glycine results in an increase in the pH of the gastric contents and thus in a smaller concentration gradient which should reduce the back diffusion possibly to an insignificant level. As mentioned above indirect estimates of C_0 in intact stomachs must be considered a little uncertain since it is not possible to tie off the cardia and pylorus. Uranine was injected intravenously before the instillations (see page 15) to detect whether duodenal regurgitation occurred. With this precaution 30–50 ml of glycine was instilled into the stomach of one of the dogs (L.) during histamine induced steady

gastric secretion The H+ ion concentration gradient was calculated to be diminished about 60–120 times by the glycine The gastric contents were removed about ten minutes after instillation The volume increment was calculated directly both from the difference between volume removed and volume instilled and also by determination of the final glycine concentration after a formal titration according to SØRENSEN (1908) The latter procedure also gives a check of the total recovery of the glycine (cf LINDE TEORELL and ÖBRINK 1947) Most experiments were spoiled by extragastric dilution and losses of glycine C_0 could be calculated from four experiments and a mean value of 163 5 mN was obtained This value agreed rather well with the mean value of 161 6 from the secretion experiments in the same dog (calculated from the Eqs 2 1 and 2 2) On account of the experimental difficulties the instillation experiments were considered not suitable for human subjects

Comments

The rather good fit of both Eq 2 1 and Eq 2 2 with the experimental data justifies the calculation of C_0 by either of these equations In Table 2 I it can be seen that the sum of the least squares Σd^2 is numerically smaller when using Eq 2 2 than with Eq 2 1 (except for H C) This should mean that the values of C_0 and k obtained from Eq 2 2 should be more representative All dogs gave higher values than the highest C_0 of the human subjects (Table 2 I)

This demonstrates that the primary acidity in man is lower than in the dog There seemed to be a tendency for the human primary acidity to be higher with increasing age but the material is rather too small for the significance of this to be assessed

The secondary acidity [H+] in Eq 2 1 and Eq 2 2 according to the diffusion theory is dependent on the magnitude of the permeability coefficient k This means for any given value of the primary acidity the k value and secondary acidity vary inversely In Table 2 I the highest acidity measured in any experiment for each human subject and dog is given The lowest of the highest [H+] values measured in the dogs was 12 mN greater than the highest value in the human subjects

If the k values were found to be of a similar magnitude it should be valid to compare the acidity for any particular secretion rate v

(for instance $v = 2.5$ ml/min) between man and dog In Table 2 I it is shown that this *comparison indicates a higher acidity in the dog*

The highest acidity values reported in man are in mN 140 FIENSBORG (1944), 140 HANSON GROSSMAN and IVY (1948) 149 NORDGREN (1958) 150 KAIJSER (1943) 150 HIRSCHOWITZ (1961), 152 8 IHRE (1938) and 154 WELIN and FRISK (1936)

In dogs the highest acidity values observed are 155 HANSON GROSSMAN and IVY (1948) 158 MARKS KOMAROV and SHAY (1958) 160 THULL and REHM (1956) 160 MARKS KOMAROV and SHAY (1960) and 161 ÖBRINK (1948) For comparison acidities of 167 GUDIKSEN (1950) and about 170 LINDE (1950) have been found in cats It thus seems

(1) *That the primary acidity in man is lower than in dog and*
(2) *That the acidity regulation is qualitatively the same for both man and dog and the 'diffusion theory' fits in with the relationship observed between acidity and secretion rate*

The observation that man and dog have different primary acidities is important because it follows that man and dog should have a *different ion composition of the 'primary juice'* and this will be discussed in a later part of this investigation

The permeability coefficient, λ

As the sum of the least squares (Σd see Table 2 I) is smaller when using Eq 2 2 than Eq 2 1 (except for subject H C) the λ values (like the C_0 values) calculated from Eq 2 2 can be considered more representative NORDGREN (1958) in man claimed to have found a relationship between the permeability coefficient λ and the maximal secretion rate although the correlation was *not very high* *In this* material when λ was plotted against the maximal secretion rate a_1 (see previous chapter) *no certain correlation* was found but this may be due however to the rather small number of subjects who were *studied*

It is evident that at submaximal gastric secretion rates a low λ value will yield a higher acidity (i e the secondary acidity) than will a high λ value (the primary acidity is assumed to be invariant) A

subject with a low k value thus could be said to have a *hyperacidity* while a subject with a high k value would be *hypoacid*

If only the acid output is measured the terms hypersecretion (see previous chapter) and hyperacidity are more difficult to interpret Hyper and hypoacidity are thus defined in terms of the acidity at a certain secretion rate It can be estimated from the k value (see Eq 2 1 and Eq 2 2)

(for instance $v=2.5$ ml/min) between man and dog In Table 2 I it is shown that this comparison indicates a higher acidity in the dog

The highest acidity values reported in man are in mN 140 FIENSBORG (1944) 140 HANSON GROSSMAN and IVY (1948) 149 NORDGREN (1958) 150 KAIJSER (1943) 150 HIRSCHOWITZ (1961) 152 8 IHRE (1938) and 154 WELIN and FRISK (1936)

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(1) *That the primary acidity in man is lower than in dog and*
(2) *That the acidity regulation is qualitatively the same for both man and dog and the diffusion theory fits in with the relationship observed between acidity and secretion rate*

The observation that man and dog have different primary acidities is important because it follows that man and dog should have a different ion composition of the primary juice and this will be discussed in a later part of this investigation

The permeability coefficient, k

As the sum of the least squares ($\Sigma\, d^2$ see Table 2 I) is smaller when using Eq 2 2 than Eq 2 1 (except for subject H C) the k values (like the C_0 values) calculated from Eq 2 2 can be considered more representative NORDGREN (1958) in man claimed to have found a relationship between the permeability coefficient k and the maximal secretion rate although the correlation was not very high In this material when k was plotted against the maximal secretion rate a_1 (see previous chapter) no certain correlation was found but this may be due however to the rather small number of subjects who were studied

It is evident that at submaximal gastric secretion rates a low k value will yield a higher acidity (i e the secondary acidity) than will a high k value (the primary acidity is assumed to be invariant) A

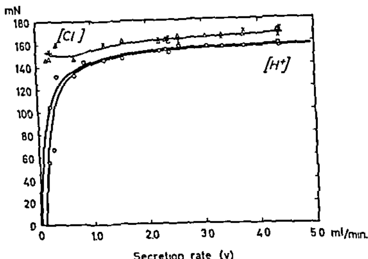


Fig 3 1 The variation of [Cl] in gastric secretion at diff rent steady rates (Dog M) The ac dity c urves are the same as in Fig 2 1 [Cl] is represented by unfilled triangles The crosses represent the sum of [H] [K] and [Na] The chlorid c rve was drawn by eye

Although the chloride concentration and output thus have been correlated with secretion rate in pouch dogs the situation in the intact stomach of dogs and man has not been investigated extensively and it was considered of interest to study further this problem

Results

Figs 1 1 and 1 3 show the variations of the chloride concentration in typical experiments The steady state values of [Cl⁻] and [H⁺] were plotted against the corresponding secretion rates in Fig 3 1 (dog M) and Fig 3 2 (subject K G K) It can be seen that [Cl⁻] (open triangles) approximately equals the sum of [K⁺]+[Na⁺]+[H⁺] (crosses) No attempt was made to fit a regression equation to the chloride values since they appear to represent the sum of all the ca tions

The net output of Cl⁻ vCl is therefore also a measure of the net output of the cations In Fig 3 3 Cl⁻ output is plotted against the secretion rate v As can be seen (Fig 3 3) this is apparently a straight line except for the lowest v values

CHAPTER 3

The Relation between the Chloride Concentration and Rate of Gastric Secretion

Chloride is the predominant anion and is a satisfactory index of the sum of cations in the gastric secretion

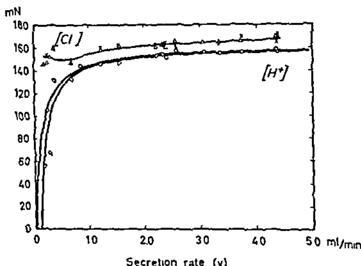
The concentration of the hydrogen ion is always less than that of chloride the excess of which is often referred to as neutral chloride

The chloride concentrations (both total and neutral) have been related to other parameters of the gastric secretion (e g the acidity and the secretion rate) *Rectilinear* relationships have been claimed between

(1) total chlorides and acidity in pouch dogs HOLLANDER (1932) GRAY BUCHER and HARMAN (1941)

(2) neutral chlorides and acidity HOLLANDER (1932) GRAY BUCHER and HARMAN (1941) in dogs and FISHER and HUNT (1950) in man using IHRE s data (1938) Through extrapolation to $[H^+]=0$ the neutral chlorides were calculated as 130–140 mN IHRE (1938) in man however taking in account back diffusion concluded that there was a curvilinear relation between total chlorides and acidity

TEORELL (1933 1939 1947) found that for chloride the results of model experiments and instillation experiments *on cats were in agreement with the predictions of his diffusion theory i e the relationship* between total chloride and acidity was *curvilinear* He also confirmed the existence of the predicted temporary dip in the chloride concentration in instillation experiments GRAY BUCHER and HARMAN *(1941) concluded there was a hyperbolic relation* between secretion rate and total chloride concentration They however had no analytical data at the very low secretion rates under which conditions in pouch dogs ÖBRINK (1948) and LINDE and ÖBRINK *(1950) did not find any* great $[Cl^-]$ decrease and concluded that these results supported the diffusion theory A rectilinear correlation between the chloride output and the secretion rate was found by GRAY BUCHER and HARMAN (1941)



Fi 3 1 The variation of [Cl] in gastric secretion at different steady rates (Dog M) The ac dity curves are the same as in Fig 2 1 [Cl] is represented by unfilled triangles The crosses represent the sum of [H+] [K] and [Na+] The chlo de curve was drawn by eye

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Figs 1 1 and 1 3 show the variations of the chloride concentration in typical experiments The steady state values of [Cl⁻] and [H+] were plotted against the corresponding secretion rates in Fig 3 1 (dog M) and Fig 3 2 (subject K G K) It can be seen that [Cl⁻] (open triangles) approximately equals the sum of [K+]+[Na+]+[H+] (crosses) No attempt was made to fit a regression equation to the chloride values since they appear to represent the sum of all the cations

The net output of Cl⁻ vCl is therefore also a measure of the net output of the cations In Fig 3 3 Cl⁻ output is plotted against the secretion rate v As can be seen (Fig 3 3) this is apparently a straight line except for the lowest v values

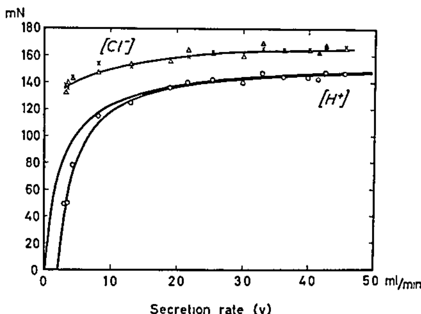


Fig 3 2 The relationship between the rate of gastric secretion and [Cl⁻] in man (Subject K G K) The acidity curves are the same as in Fig 2 2 The notations are is the same as in Fig 3 1

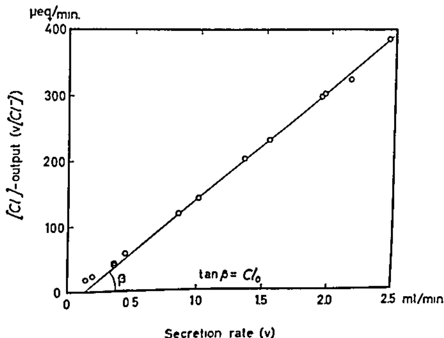


Fig 3 3 Rate of gastric secretion plotted against output of total chlorides v [Cl⁻] per unit time The continuous line was drawn by eye through the experimental points The slope of the continuous line tan β should equal Cl_0 i e the chloride concentration in the primary secretion see text (Subject G I)

Discussion

The values of the total chloride concentration in secretion experiments have been found to range between about 140 and 170 (mN) in man (IHRE 1938) and in pouch dogs about 140–170 (mN) (ÖBRINK 1948)

The maximal chloride concentration in man was found to reach a level of about 170–180 (mN) (HIRSCHOWITZ 1961) The equilibrium Cl^- concentration as found by instillation experiments at quasi zero secretion rates tends to the values of about 140–150 (mN) TEORELL (1933) or 140 (mN) KAIJSER (1943) in cats and in man about 125–135 (mN) IHRE (1938) or 120 (mN) KAIJSER (1943) In the present instillation experiments [Cl^-] values of about 140 (mN) were obtained Difficulties in excluding extragastric admixture in man may explain the variations found In the present secretion experiments the range for the chloride concentration was in man 119–167 (mN) and in dog 154–176 (mN) Both in this material and in the work cited above there was no significant difference for the upper limit of the total chloride concentration between man and dog

In order to calculate the primary chloride concentration Cl_0 i e the concentration as the juice emerges from the secretory cell the output of the Cl^- per minute (vCl) was plotted against the secretion rate v Fig 3 3 As can be seen there is a linear relationship except at the lowest v values which means that the Cl^- concentration tends to change very little except at very low secretion rates HIRSCHOWITZ (1960) also found linearity between Cl^- output and the gastric osmolar clearance (= the secretion rate times the quotient osmotic pressure of gastric juice/plasma) The straight part of the curve may be considered as an asymptote and its slope would then be a measure of Cl_0 which has been calculated in Table 3 I There was no obvious difference between man and dog (Table 3 I)

Comparison of [Cl] in the 'primary secretion' of man and dog

A comparison between the Cl_0 values and the earlier calculated mean values of the primary acidity C_0 from Eq 2 1 and Eq 2 2 (see chapter 2) should give information about the amount of neutral

TABLE 3 I *Data of the calculation of the total [Cl⁻] and the neutral [Cl⁻] in the primary secretion*

Subject	Number of Experiments	Cl_0 mN	C_0 (mean from Eqs 2 1 and 2 2)	Cl_0–C_0 (mean) mN	
K G K	15	183	155 7	27 3	
B I	18	175	155 9	19 1	
H C	10	166	147 0	19 0	
R N	12	158	128 3	29 7	
G L	13	165	145 7	19 3	$M = 22\ 9$
Dog L	8	165	161 6	3 4	
Dog M	17	168	161 6	6 4	$M = 4\ 9$

chlorides present in the primary secretion There seems to be a striking difference between man and dog in this respect a value of about 20–25 mN was calculated for man and about 5 mN for the dog It thus appears that man has a lower acidity but a higher neutral chloride concentration in the primary secretion' compared with the dog

CHAPTER 4

The Relation between the Sodium Concentration and Rate of Gastric Secretion

At higher rates of gastric secretion the sodium concentration is lower in contrast to the acidity which is higher HOLLANDER (1952) showed in pouch dogs an inverse rectilinear relationship between [Na+] and [H+] In man RIDDELL STRONG and CAMERON (1960) and HIRSCHOWITZ (1961) found that a curvilinear equation fitted their data better GRAY and BUCHER (1941) in pouch dogs concluded that the [Na+] was a hyperbolic function of the secretion rate From his diffusion hypothesis TEORELL (1947) derived the relation

$$Na = \frac{S_0}{\frac{v}{r}+1} \qquad (4\ 1)$$

where Na = the sodium concentration observed (mN)
v = the rate of gastric secretion (ml/min) (in Teorell s notation q)
S_0 = the sodium concentration (mN) when $v = 0$ and
r = the permeability coefficient for Na^+ (ml/min)

In model experiments he found that Eq 4 1 fitted the experimental data rather well

LINDE and ÖBRINK (1950) in pouch dogs found Eq 4 1 fitted their results in secretion experiments The purpose of this study was to relate [Na+] with the secretion rate in intact stomachs of man and dog

Results

The relationship between sodium concentration and acidity and secretion rate during steady state conditions with continuous histamine stimulation is illustrated in the dog in Fig 1 1 and man in Fig 1 3 The different steady state values of [Na+] and [H+] from each single experiment are plotted against the corresponding rates of gas

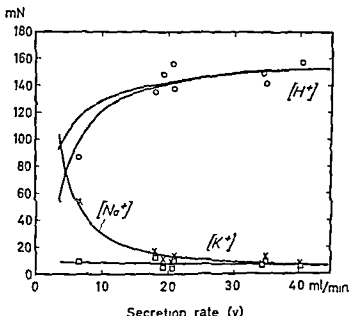


Fig 4 1 The relationship between the rate of gastric secretion and [Na⁺] and [K⁺] during steady state conditions The extrapolation to $v = 0$ was considered uncertain because of the few experimental values at low rates of secretion The regression curve for [Na⁺] (marked with crosses) was obtained from Eq 4 1 The [K⁺] secretion rate relationship (unfilled squares) is represented by a straight line (Dog L)

tric secretion in the same subject Fig 4 1 (dog L) and Fig 4 2 (man G L) The inverse relation between [Na⁺] and [H⁺] is obvious

The scattering of the results is bigger at lower than at higher secretion rates (Fig 4 2) This may be due to the difficulties in controlling extragastric admixture

The regression curve for [Na⁺] was obtained by using the method of least squares with Eq 4 1

Estimation of the parameters in Eq 4 1

The form of the relationship in Eq 4 1 is of such a kind that a direct regression analysis on the basis of least squares principle will involve certain difficulties If instead the logarithms of both sides are taken a linear relationship is obtained between the variables log Na and log $(v + r)$

$$\log \mathrm{Na} = \log rS_s - \log (v + r) \qquad (4\ 2)$$

The estimation of the parameters can be performed in the following steps on the basis of the above equation

(1) Let the parameter r have a plausible *a priori* value
(2) Estimate $\log rS_s$ according to the L S principle
(3) Calculate the sum of the squared residuals

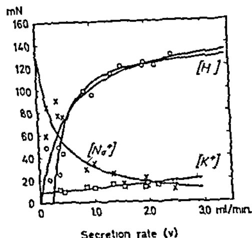


Fig 4 2 The same relations as in Fig 4 1 (Subject G L)

(4) If the above steps have been made for several values of r it is possible to plot the sum of the squared residuals against the r values and thus find graphically that value of r which makes the sum of the squared residuals a minimum

The regression curves for the acidity were derived from Eq 2 1 (the curve passing through the origin) and Eq 2 2

The curves obtained from man and gastric fistula dogs were in general similar

Discussion

Eq 4 1 is a hyperbolic function with an asymptote ($r = -v$) parallel with the y axis A small error in r means a small displacement of the asymptote along the x axis which would result in a rather large error in the value of S_0 When applying the method of least squares it is sometimes possible to get negative S_0 values This is especially likely to occur in subjects with sparse data from experiments with low secretion rates (Table 4 I) A trial was made therefore to estimate S_0 in another way Log $[Na^+]$ was plotted against v for the whole material and extrapolated to $v = 0$ (Table 4 I) In those cases where S_0 could be calculated from the hyperbolic Eq 4 1 a check on the validity of this second procedure was obtained The agreement was acceptable

In the acidity secretion rate relation the acidity curve appeared to

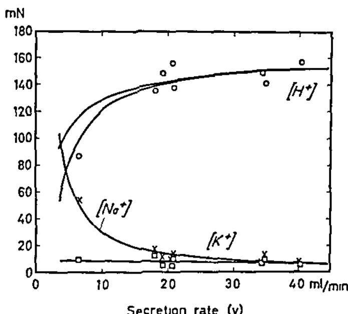


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(3) Calculate the sum of the squared residuals

CHAPTER 5

The Relation between the Potassium Concentration and Rate of Gastric Secretion

Gastric secretion always contains alkali ions and at low secretion rates sodium predominates At higher secretion rates sodium dimin ishes progressively being largely replaced by H^+ ions and the greater part of the alkali ions then consist of potassium If it is assumed that the primary secretion does not contain any Na^+ the increase in $[Na^+]$ with decreasing secretion rate can be explained entirely by diffusion of Na^+ through the stomach wall As potassium however does not decrease (but instead at least in man increases) with increas ing secretion rate the relation between secretion rate and potassium similarily cannot be explained in the same way as for sodium It seems likely that potassium also is secreted in the primary secretion (cf LINDE and ÖBRINK 1950) in contrast to sodium

The relations between $[K^+]$ and $[H^+]$ and between $[K^+]$ and secretion rate

Correlations between the potassium concentration and the acidity or the rate of gastric secretion have been claimed by several authors and these relationships have been a matter of much discussion After a single subcutaneous injection of histamine SAEMUNDSSON (1948) WERTHER PARKER and HOLLANDER (1960) in man and HOLLANDER (195-) and HOLLANDER and COLCHER (1960) in pouch dogs (the last named also gave repeated subcutaneous injections of histamine) did not find any constant relationship between $[K^+]$ and $[H^+]$ or $[K^+]$ and secretion rate On the other hand a definite correlation between $[K$ $]$ and $[H$ $]$ was found in human subjects who had received a single subcutaneous injection of histamine (RIDDELL, STRONG and CAMERON 1960) and in cats after continuous intravenous injection of histamine (BLAIR HARPER HARRIS REED and WILKINSON 1960 and BLAIR HARPER and LASSEN 1961) Further RIDDELL, STRONG and CAMERON

TABLE 4 I *Calculated data from the relationship between rate of gastric secretion and [Na⁺]*

Subject	Number of experiments	r ml/min	S_0 mN	S_0 graphically mN
K G K	15	−0 1	−166 [illegible]	1[illegible]0
B I	18	−0 4	−39 8	135
H C	10	0 4	113 5	96
R N	12	1 1	84 9	85
G L	13	0 3	131 9	135
Dog L	8	−0 1	−267 9	87
Dog M	17	−0 1	∞	130

intersect the positive x axis in some cases (Eq 2 2) The reason for this may be incomplete mixing of the gastric contents which is not accounted for in Eq 4 1 If Eq 2 2 is a satisfactory description of the acidity secretion rate relationship then physically impossible values of r (negative) and S_0 (tending to infinity) may result if Eq 4 1 is used to analyse the [Na^+] results

The range for the [Na^+] values was 90 1 to 2 9 (mN) in man and 82 5 to 2 5 (mN) in dog i e there was a great similarity between the species Furthermore the decline in [Na^+] with increasing secretion rates (Fig 4 2) together with the small sodium concentration found at the higher secretion rates supports the diffusion theory and the results do not contradict the idea that [Na^+] $\rightarrow 0$ when t becomes very large (i e that the primary secretion ' contains no sodium)

CHAPTER 5

The Relation between the Potassium Concentration and Rate of Gastric Secretion

Gastric secretion always contains alkali ions and at low secretion rates sodium predominates At higher secretion rates sodium diminishes progressively being largely replaced by H^+ ions and the greater part of the alkali ions then consist of potassium If it is assumed that the primary secretion does not contain any Na^+ the increase in $[Na^+]$ with decreasing secretion rate can be explained entirely by diffusion of Na^+ through the stomach wall As potassium however does not decrease (but instead at least in man increases) with increasing secretion rate the relation between secretion rate and potassium similarly cannot be explained in the same way as for sodium It seems likely that potassium also is secreted in the primary secretion (cf LINDE and ÖBRINK 1950) in contrast to sodium

The relations between $[K^+]$ and $[H^+]$ and between $[K^+]$ and secretion rate

Correlations between the potassium concentration and the acidity or the rate of gastric secretion have been claimed by several authors and these relationships have been a matter of much discussion After a single subcutaneous injection of histamine SAEMUNDSSON (1948) WERTHER PARKER and HOLLANDER (1960) in man and HOLLANDER (1952) and HOLLANDER and COLCHER (1960) in pouch dogs (the last named also gave repeated subcutaneous injections of histamine) did not find any constant relation hip between $[K^+]$ and $[H^+]$ or $[K^+]$ and secretion rate On the other hand a definite correlation between $[K^+]$ and $[H^+]$ was found in human subjects who had received a single subcutaneous injection of histamine (RIDDELL STRONG and CAMERON 1960) and in cats after continuous intravenous injection of histamine (BLAIR HARPER HARRIS REED and WILKINSON 1960 and BLAIR HARPER and YASSIN 1961) Further RIDDELL STRONG and CAMERON

(1960) found a positive linear relationship between [K^+] and rate of gastric secretion in man. In pouch dogs GRAY and BUCHER (1941) after repeated subcutaneous injections of histamine and LINDE and ÖBRINK (1950) after continuous intravenous injection of histamine got a fairly constant [K^+] at varying secretion rates.

HIRSCHOWITZ (1961) found that the relationship between K^+ and H^+ was disturbed by atropine or $NaHCO_3$. After parenteral administration of atropine or $NaHCO_3$ in man during histamine stimulation there was a fall in [K^+] while [H^+] *remained unchanged. The effect* of $NaHCO_3$ cannot be fully evaluated as the acid base status was incompletely analyzed.

In conclusion it may therefore be said that there is thus much evidence of a correlation between [K^+] and secretion rate both with and without steady state conditions but on the other hand there has been objection to the existence of such a correlation.

The situation is therefore somewhat confused and more information about the potassium concentration in gastric secretion is desired. Furthermore in view of a possible connection with the primary secretion of acid and the difference between the primary acidity of man and *dog (chapter 2) potassium deserves close attention. It is here relevant* to recall that potassium has been considered to be secreted along with the hydrogen ions in the primary secretion (HEINZ and ÖBRINK 1954).

To obtain more information and comparable experimental conditions a method giving steady state conditions is preferable and was therefore used in this study in both man and in intact stomachs of dogs. As discussed in an earlier chapter only repeated subcutaneous injections or continuous intravenous injections (but not a single subcutaneous injection) of histamine can give steady *state conditions*.

Results

Fig. 11 (dog) and Fig. 13 (man) illustrate experiments with continuous intravenous injection of histamine. During a steady state in the rate of gastric secretion the potassium *concentration remained* fairly constant. The relationship between secretion rate and [K^+] is shown in *Fig.* 41 *in* dog and Fig. 42 in man. The experimental

TABLE 5 I *Data obtained from the [K+] secretion rate relationship*

Subject	n	[K+] for v=0 mM	d[K]/dv mM min/ml	[K+] for v=4 mM	Cl-C mM	[K+] in gastric secretion range mM
K G K	15	8 7	1 74	15 7	27 3	7 9-16 9
B I	18	7 3	3 8	20 4	19 1	8 8-19 3
H C	9	11 6	2 19	20 4	19 0	11 1-18 9
R \	10	7 9	2 49	19 1	29 /	9 3-18 0
G L	10	8 1	4 19	24 9	19 3	11 2-19 1
D g L	8	9 4	−1 59	6 2	3 4	5 0-11 8
Dog M	16	5 9	−0 35	5 2	6 4	3 7-8 1

values of [K+] in the gastric secretion for each subject is given in Table 5 I The variation of [K+] in the dog experiments in this material is bigger than LINDE and ÖBRINK (1950) found in pouch dogs where however the experimental conditions as regards extragastric admixture must be more favourable

The [K+] during steady secretion rate in the present dog experiments tended to decrease slightly with higher rates of gastric secretion This was in contrast to man where [K+] was always greater with higher secretion rates

Because the volumes of the gastric juice were sometimes too small [K+] could not be determined in these samples with sufficient accuracy

Discussion

Calculation of the regression line

Starting with the assumption that potassium is secreted by the parietal cells and that the mucosa is permeable to it HEINZ and ÖBRINK (1954) derived an equation which indicated that there should be a curvilinear relationship between secretion rate and [K+] The equation was applied to this material but it is rather laborious to use The analysis showed that within the range of secretion rates used the curvilinearity was insignificant and in this case an approximately straight line was obtained An equally good fit for the relation be

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Results

Fig 1 1 (dog) and Fig 1 3 (man) illustrate experiments with continuous intravenous injection of histamine During a steady state in the rate of gastric secretion the potassium concentration remained fairly constant The relationship between secretion rate and [K+] is shown in Fig 4 1 in dog and Fig 4 2 in man The experimental points fitted well to a straight line The range of the steady state

TABLE 5 I *Data obtained from the [K⁺] secretion rate relationship*

Subject	n	[K⁺] for $v = 0$ mN	$\frac{d[K^+]}{dv}$ mN $\frac{min}{ml}$	[K⁺] for $v = 4$ mN	Cl – C mN	[K⁺] in gastric secretion range mN
K G K.	15	8 7	1 74	15 7	3	7 9–16 9
B J	18	7 3	3 °8	20 4	19 1	8 8–19 3
H C	9	11 6	– 19	20 4	19 0	11 1–18 9
R N	10	7 9	° 7J	1J 1	29	9 3–18 6
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tween [K+] and secretion rate was obtained with the linear regression line calculated by the method of least squares In Table 5 I the values for the y axis intercept (i e [K+] for $t=0$) and the slope of the straight line here denoted as d[K+]/dt are given As can be seen (Fig 4 1 and Fig 4 2) [K+] shows a good correlation with the rate of gastric secretion using a rectilinear relationship

Magnitude of [K+] in the "primary secretion"

Since a curvilinear relationship with a maximal [K+] value was not calculated for the relationship between [K+] and the secretion rate the [K+] value corresponding to the infinitely high secretion rate can not be calculated from the straight regression line To estimate the [K+] in the primary secretion the values of [K+] corresponding to a secretion rate of 4 0 ml/min are given in Table 5 I The value of 4 0 ml/min was chosen as an estimate of the maximal secretion rate it being an approximation of the mean of the a_1 values (see chapter 1) A comparison with the neutral chloride concentration calculated in the primary secretion (see chapter 3) is given in Table 5 I and shows that the higher [K+] in man can account for the higher neutral chloride concentration and the lower primary acidity in man compared with the dog

It was shown in chapter 3 that the primary chloride concentration was not different in man and dog Chloride is practically the only anion in the gastric secretion which therefore is equiosmolar in dog and man The higher [K+] in man could thus not be accounted for by a higher osmolarity in the primary secretion This supports the *rather essential conclusion that the primary secretion in man* though of the same osmolarity has a different composition from that of the dog having *a higher [K+] and a correspondingly lower primary acidity*

The origin of K+ in the gastric secretion

The potassium concentrations calculated for $t=0$ (i e at secretory arrest) in man and dog were not significantly different (Table 5 I) This is of some interest because it suggests that at secretory arrest potassium tends to reach the same concentration in both man and dog It seems possible that at secretory arrest the intragastric [K+] is in a steady state between the surrounding compartments i e between the intracellular and the plasma potassium

The plasma potassium concentration in dogs (PROSSER 1950) is not very different from that in man (NADLER 1953 BERNSTEIN 1954) If the intragastric potassium (secretory arrest) is considered as being in a compartment lying in contact with both the intracellular and extracellular (plasma) spaces the similarity of the *intragastric* and *extra*cellular (plasma potassium) concentrations in man and dog might perhaps mean that the *intra*cellular [K^+] values are similar in man and dog

The parietal cells are only part of the total cell population of the mucosa This means that [K^+] in the parietal cells is not necessarily the same as the average for the whole cell population In fact DAVENPORT and ALZAMORA (1962) found differencies in [K^+] between parietal cells and the surface epithelial cells in the frog gastric mucosa It is therefore possible that although a rather similar average [K^+] in the whole cell population in man and dog is likely (see discussion above) the [K^+] values for the parietal cells may be different in dog and man As potassium probably to a large extent is derived from the parietal cells (see chapter 7) such a difference in the two species may cause different concentration of K^+ in the primary juice In fact at higher rates of gastric secretion the difference in [K^+] is manifest

Another explanation for the difference of the primary K^+ concentration might be different rate constants for the secretion of K^+ from the parietal cells In such a case we do not need to assume different intracellular concentrations in the parietal cells of man and dog Such differences in rate constants are not unlikely as was shown by BURGEN (1956) to occur in salivary glands when the degree of stimulation changed

In short the study supports the view that the primary secretion contains potassium and in a higher concentration in man than in dog This [K^+] is dependent on the secretion process Further [K^+] in the stomach contents at secretory arrest is not different between the species The findings are good reason for further studies of the kinetics of potassium transport in the gastric juice

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CHAPTER 6

The Effect of Histamine on Potassium Concentration in Blood, *in vitro* and *in vivo* Experiments

After continuous intravenous injection of histamine there was a significantly higher output of potassium in the gastric secretion of man than in dog At secretory arrest [K+] was calculated to be not significantly different between man and dog (chapter 5)

This study attempts to analyze the mechanisms underlying the higher [K+] in gastric juice in man during secretion The aim was to study if the higher gastric potassium concentration obtained in man was a passive process i e due to simple diffusion equilibrium with plasma potassium caused secondarily by an increase in plasma potassium (from systematic potassium liberation induced by histamine)

As discussed in chapter 5 plasma [K+] appears to be similar in both species but this is no measure of the kinetics of potassium exchange It is quite possible that in man more potassium is liberated from different parts of the body and then transported to the stomach mucosa

It has been suggested that histamine may act by liberating potassium from tissues In cats histamine has been shown to increase the plasma potassium concentration (MacMillan 1956 1957) and this effect could be antagonised by antihistamines (promethazine or pyrilamine)

Similar results have been reported in rabbits and guinea pigs (Ambrus and Ambrus 1952) Liberation of K^+ from tissues after histamine administration was found by Parrot Thouvenot and Longuevalle (1952 1953) for example on guinea pig intestine in cat red blood cells (MacMillan and Vane 1956) and in cat striated muscle (MacMillan 1955)

It has been shown in isolated frog gastric mucosa during secretion that an increase of [K+] in the nutrition fluid gave an increase in the net transport of K^+ to the secretory side (*Harris Frank and Edelman*, 1958) With the [K+] on the secretory side 4 0 mN/l higher

than on the blood side there was still a moderately reduced net transport across to the secretory side

It seems justified therefore to consider whether the higher K^+ output in gastric secretion in man depends on a higher extragastric K liberation than in dog At this point it should be mentioned that $[K^+]$ in red blood cells of the dog was found to be 10 mN BERNSTEIN (1954) 9 mN PONDER (1948) and 8 7 mN KERR (1937) compared with the much higher values in man e g 106 mN BERNSTEIN (1954) 100 mN PONDER (1948) and 99 2 mN LANS STEIN Jr and MEYER (1952) Thus in marked contrast to the dog there is clearly a not unimportant potential potassium pool in the red cells of man

In this study both *in vitro* experiments with whole blood and *in vivo* determinations of plasma $[K^+]$ before and during histamine administration were made The aim of the *in vitro* experiments was to study whether potassium was liberated from the potassium rich erythrocytes of man (measured as an increase in plasma $[K^+]$) after addition of histamine The aim of the *in vivo* experiments was to study whether there was any liberation of K^+ from other parts of the body

Methods

Blood was collected in tubes treated with dimethyldichloresilane (Repelcote* Hopkins and Williams Ltd) and containing a small amount of heparin Plasma was obtained by centrifugation Dilutions were made with 0 1 ammoniated water to solubilise particulate material as according to BARER and GAFFNEY (1957) Potassium was determined flamephotometrically 2 ml of plasma was diluted 1 5 with 7 ml ammoniated water and 1 ml 100 mN LiCl $[K^+]$ *in the* red blood cells was calculated from the hematocrit and potassium concentrations in whole blood (hemolysed with ammoniated water) and plasma No correction was made for trapped plasma

In vitro experiments

Histamine dihydrochloride in differing amounts (see Table 6 I) was added to the blood samples which were then shaken for about 15 minutes together with the controls (without any histamine added) before centrifugation The blood used was up to 5 days old

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sample as the histamine treated cells The $[K^+]$ difference with and without histamine was calculated The mean difference including all histamine levels was for man +0 1 mN ($n=9$ t test $t=1\ 51$ $P>0\ 1$) and for dog −0 1 mN ($n=8$ t test $t=1\ 01$ $P>0\ 1$) There was no tendency of increase in $[K^+]$ with increasing amounts of histamine

Thus in the concentration range of histamine used and in the absence of an antihistamine an *in vitro* increase of plasma potassium concentration was not detected

In vivo experiments

The mean difference between plasma $[K^+]$ before and during the histamine stimulation was −0 289 mN ($n=9$ t test $t=2\ 75$ and $0\ 05>P>0\ 02$) As in the *in vitro* experiments there was no successive increase in plasma $[K^+]$ with increasing injection rates of histamine Thus there was no evidence of an increase in plasma $[K^+]$ during histamine stimulation Further work would be required to show if the apparent decrease had any significance Table 6 II shows the $[K^+]$

TABLE 6 II *Measured values of $[K^+]$ in gastric secretion*

Subject	Without histamine stimulation	With histamine stimulation
B I	8 8	19 4
H C	11 1	18 9
R N	9 3	18 6
G L	11 2	18 1
Dog L		11 8
Dog M		8 1

TABLE 6 III *Examples of changes in plasma $[K^+]$ and $[K^+]$ in secretion before and during histamine injection*

Subject	$[K^+]$ in plasma (mN)		$[K^+]$ in gastric secretion (mN)		Injection rate of histamine µg/kg hour
	Before histamine injection	During histamine injection	Before histamine injection	During histamine injection	
B I (4)	5 0	5 [illegible]	[illegible]9	18 7	17 2
B I (5)	5 3	5 0	9 1	14	[illegible]3

Table 6 I *Experiments (in vitro) in man and dog showing [K⁺] in blood plasma without and with histamine addition*

	Man		Dog	
	Histamine conc µg/100 ml blood	$[K^+]$ mM	Histamine conc µg/100 ml blood	$[K^+]$ mM
	0.0	4.3	0.0	6.1
	12.5	4.2	12.5	5.7
	25.0	4.3	25.0	6.1
	67.5	4.3	67.5	5.8
	125.0	4.2	125.0	6.1
In red cells	–	97.8	–	7.1

In vivo experiments

Blood was taken from a peripheral vein before the histamine injection and during a steady gastric secretion rate induced by histamine. In these experiments the antihistamine chlorpheniramine was given to the subject as in the other experiments with gastric secretion (see earlier chapters).

In both types of experiments the effect of histamine administration on the plasma $[K^+]$ was compared.

Results

In vitro experiments

In pouch dogs ÖBRINK (1948) found a linear relationship between the rate of continuous intravenous histamine injection and the plasma concentration of histamine. For injection rates giving an almost maximal rate of gastric secretion plasma concentrations of 60 µg/100 ml of histamine dihydrochloride were obtained. With 48 µg/100 ml of histamine base in plasma EMMELIN, KAHLSON and WICKSELL (1941) observed pronounced pharmacological systemic effects on dogs. It was therefore decided to use blood concentrations of histamine dihydrochloride from 12.5 to 125 µg/100 ml in this study. No antihistamine was used. In *Table 6 I* results from human and canine blood are shown. In all cases histamine controls were of the same blood

sample as the histamine treated cells The [K+] difference with and without histamine was calculated The mean difference including all histamine levels was for man +0 1 mN ($n = 9$ t test $t = 1\ 51$ $P > 0\ 1$) and for dog −0 1 mN ($n = 8$ t test $t = 1\ 01$ $P > 0\ 1$) There was no tendency of increase in [K+] with increasing amounts of histamine

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Subject	Before histamine injection	During histamine injection	Before histamine injection	During histamine injection	Injection rate of histamine µg/kg hour
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B I (5)	5 3	5 0	9 1	14	3

values in the gastric secretion from some experiments in man and dog during histamine stimulation Table 6 III shows in two experiments in man (B I) with the plasma [K^+] and the [K^+] in gastric secretion before and during histamine stimulation

Discussion

No evidence of an increase in plasma [K^+] was found either in blood treated with histamine *in vitro* or in blood in *in vivo* experiments after histamine administration As a significant difference between *plasma* [K^+] in man and dog has not been established the higher [K^+] in human gastric secretion during histamine stimulation can not be *explained by a higher plasma* [K^+] *which might give secondarily* a higher [K^+] in gastric secretion in man

A higher potassium *liberation in man might not be reflected* however by an increase in plasma [K^+] if for example the rate of potassium loss from the blood was also increased pari passu This might be of importance if the potassium pools in man were of a higher concentration or were more easily liberated by histamine (if [K^+] was not different) than in dog It may be noted that antihistamines (which have been shown to block partly at least the K^+ liberation see above) were given before gastric secretion was induced by histamine

Human red cells have a higher potassium content than in dog The muscles which constitute the main storage site for K^+ in the body (FENN NOONAN MULLINS and HAEGUE 1941 FORBES 1955) have similar [K^+] values in man and dog (cf for instance HASTINGS and EICHELBERGER 1937 and EICHELBERGER and ROMA 1949) It seems not unreasonable to expect *that if histamine liberates significant* amounts of K from red cells that the K escaping from the potassium *rich human cells should have been detected* in the *in vitro* experiments

As man and dog have essentially similar K^+ concentrations in the main storage sites (predominantly the muscles) the possibility remains that K might be more easily liberated in man than in dog It should be mentioned here that at steady rates of gastric secretion the higher [K^+] in man is maintained on a fairly constant level for several hours It might be expected therefore that during such conditions a higher plasma [K^+] should be found if there was a *correspondence* of steady state [K^+] levels in gastric secretion and plasma

Although as mentioned above the KCl concentration on the nutrient side was shown to have an effect on K transport across the frog gastric mucosa the increase in intragastric [K^+] during secretion in man does not seem to arise from an increase in blood [K^+] It seems therefore possible that the *difference in intragastric [K^+] between man and dog is dependent on a local secretory mechanism in the gastric mucosa*

CHAPTER 7

The Movement of Radioactive Sodium and Potassium across the Stomach Wall

As has been described previously (chapter 5) it was found that the intragastric potassium concentration during *acid secretion is higher* in man than in dog The total intragastric cation concentration in both species was however similar and the primary acidity was shown *to be correspondingly lower in man than in dog* Thus a general liberation of potassium from the cells of the body as an effect of administered histamine was not likely to be the cause of the differences in intragastric potassium concentration in man and dog It was suggested that this difference was probably related to the local secretion process in the gastric mucosa and this motivated a study of the pathways for the transport of potassium in the gastric mucosa

The movements of radioactive potassium and sodium through the gastric mucosa were measured simultaneously so that they could be compared both during secretion and at secretory arrest

Material

Cats (weight 1 2–4 2 kg) and young dogs (weight 3 4–6 2 kg) were used in acute experiments

In two experiments with gastric secretion a Heidenhain pouch dog (weight about 20 kg) was used

Methods

Procedure

Anaesthesia In the acute experiments on cats and dogs the animals were anaesthetized first with ether *in a closed cage* *Anaesthesia* was then maintained with Nembutal® (Abott) 30–50 mg per kg body weight given intravenously or a mixture of chloralose (1 % solution

60-100 mg/kg body weight)—urethane (10 % solution 1 0-1 5 g/kg body weight) in a few cases According to STEWART (1950) MAC MILLAN and VANE (1956) barbiturate anesthesia can influence plasma [K^+] This might explain the variations in plasma [K^+] observed in the present material

The abdominal wall was opened and the stomach tied off at the cardia and pylorus (the big blood vessels passing the sphincters were left intact) A glass tube was then inserted into the pyloric end of the stomach The stomach was then emptied completely at intervals varying between about 2 and 10 minutes (see results) via a rubber tube introduced through the glass tube and the volume of the gastric contents withdrawn was estimated in a measuring cylinder

In the secretion experiments stimulation was induced with continuous intravenous injection of histamine after 8 mg chlorpheniramine (Allergisan®) had been given intravenously in a single dose

Blood samples (about 1 ml) were taken via a plastic tube (treated with heparin) inserted in a femoral artery After centrifugation 0 2 ml of plasma was used for determination of the isotope activities According to MACMILLAN and VANE (1956) 20 samples of 1 5 ml blood (at intervals of either 2 or 10 minutes) can be withdrawn from anaesthetized cats without any increase in plasma potassium The blood samples were centrifuged (for about 8 minutes at about 2000 rpm) immediately after they were taken The plasma was then removed This was done to diminish the possibility of exchange with the potassium in the red cells

Analysis

The acidity was titrated with bromthymolblue For further description of this method and the determination of Cl^- K^+ and Na^+ see page 7

Analysis of Na^{24} and K^{42}

Na^{24} and K^{42} were measured simultaneously in a two channel gamma spectrometer For details of the technique reference may be made to ÖBRINK and ULFENDAHL (1959) and BILL, ÖBRINK and ULFENDAHL (1959) The gamma ray energy for K^{42} is 1 53 MeV and for Na^{24} 1 37 and 2 76 MeV The channels were chosen so that one channel registered practically no K^{42} activity The error for the determination of Na^{24} is about 1 3 % and for K^{42} about 1 6 (20 000 cpm of the

isotopes) With lower counting rates the error becomes greater this is especially the case if there is a pronounced difference between the activities of the isotopes (DANIELSON and SJOSTRAND 1963)

The stability of the apparatus was controlled by counting standard samples of Na^{24} and K^{4} at least every hour The amounts of radio activity used were 50–70 μC Na^{24} and 50–70 μC K^{4} *The total amounts* of alkali cations injected into the animals were about 10 μEq of Na and 45 μEq of K

Results

Three types of experiments were done

I A single dose of K^{4} and Na ^{4} was given intravenously during histamine stimulation (dose about 0 5–1 0 mg/hour) to study the pattern of the appearance of the isotopes in the stomach during secretion (10 experiments)

II In two experiments the K^{4} and Na ^{4} were given intravenously without histamine stimulation (i e during secretory arrest of the stomach) but with sodium and potassium chlorides instilled in the stomach in concentrations corresponding approximately to the steady state values of the ions at secretory arrest see Table 7 I cats *4* and *5* (mean values) *This experiment was done to get information of the* isotope fluxes without any net transport of water into the stomach

III In two experiments the rate of departure from the stomach during secretory arrest was measured by the disappearance after intragastric instillation of the isotopes in a solution of the same type of composition as above see Table 7 I cats *3* and *7* (mean values)

I *The isotopes injected intravenously during gastric secretion*

Fig 7 1*a* shows the variation of the specific activities (cpm/mEq) (for practical reasons the specific activity was expressed in cpm/mEq here) of the potassium and the sodium in plasma and gastric juice after a single intravenous *dose of* K^{4} *and* Na^{24} *during continuous* intravenous injection of histamine (cat *6*) This means that a constant secretion rate (ml/min) was maintained with a constant $[H^+]$ $[Na^+]$ $[K^+]$ and $[Cl^-]$ during the whole experiment i e the rate of potassium and sodium output is constant With a single intravenous injection of K^{42} and Na ^{4} the specific activity of potassium (and to a lesser extent also of sodium) reached a *peak* about 8 minutes after the

injection Subsequently the level of the specific K activity declined and then remained at a *lower* level than the specific activity in the plasma In all experiments the specific activity of sodium also declined until it reached a level similar or a little higher than the specific activity in plasma

During gastric secretion and after the initial peak of specific activity had passed and a more or less steady level had been attained the quotient between the specific activities of potassium in gastric juice and plasma was found to be 0 33 (mean) in cats (5 animals 40 determinations) and 0 57 (mean) in dogs (5 animals 39 determinations) The corresponding values for sodium were 1 13 in cats and 1 07 in dogs Table 7 I gives the data in more detail

II *The tracers injected intravenously during secretory arrest with an approximately isotonic solution of KCl and NaCl instilled into the stomach*

In Fig 7 2 (cat 4) the stomach was in secretory arrest and 7 ml solution containing KCl and NaCl were introduced In Table 7 I data from the experiments are given In the experiments with a volume instilled in the stomach the variations in [Na^+] and [K^+] were between some samples not negligible There was however no definite trend in these concentration changes The gastric contents were then withdrawn as completely as possible at intervals of about 10 minutes A sample (0 2 ml) was then taken before reintroduction of the contents As in the experiment of Fig 7 1a K^{42} and Na^{24} were injected intravenously in a single dose A sharp initial rise of specific activity in the instillation volume was not obtained Compared with the secretion experiments the specific activity of sodium in the instilled solution increased continuously rather slowly and after 90–100 minutes had reached a level of about 20–30 % of the specific activity in the plasma Only very low specific activities of K were found intragastrically

III *The isotopes instilled in the stomach during secretory arrest*

In order to determine the rate of disappearance of K^{42} and Na^{24} from the stomach during secretory arrest 7 ml of an approximately isotonic solution (containing NaCl and KCl with K^{42} and Na^{24}) were instilled (see Table 7 I and remarks above about concentration changes) Sampling was done as above (II) at about 10 minute intervals for determination of the specific activities of potassium and sodium and

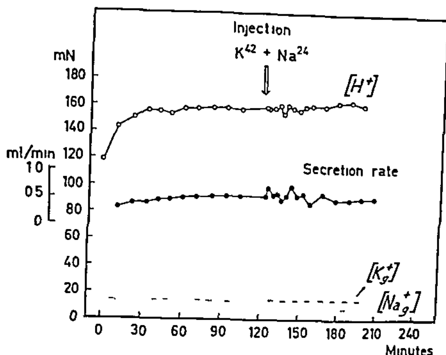


Fig 7 1a b and c Single intravenous injection of K^{42} and Na^{24} during steady rate gastric secretion (induced with continuous intravenous injection of histamine) (Cat 6) This figure (7 1 a) shows the rate of secretion and $[H^+]$ $[K^+]$ and $[Na^+]$ in the secretion

flamephotometric analysis As the fall in specific activity was exponential the logarithm of the specific activity of the e ions was plotted against the time thus giving a straight line (Fig 7 3) From two experiments the mean decrease in specific activity of potassium in the instilled solution was 36 % and for sodium 23 % after 60 minutes The specific activities in the plasma were always very low which means that the concentration gradient across the stomach was only determined by the value of the specific activity in the stomach

Discussion

I *K^{42} and Na^{24} injected intravenously during gastric secretion*

The peaks of highest specific activity in the stomach contents occurred about 8 minutes after the intravenous injection of K^{42} and Na^{24}

Fig 7 1b The *activities* of K^{42} and Na^{24} (cpm) in plasma and gastric secretion after injection of the isotopes (Cat 6)

Fig 7 1c The time course of the *specific activities* of K^{42} and Na^{24} in plasma and gastric secretion after injection of the isotopes (Cat 6)

cpm

Injection

$K^{42} + Na^{24}$

Na_p^{24}

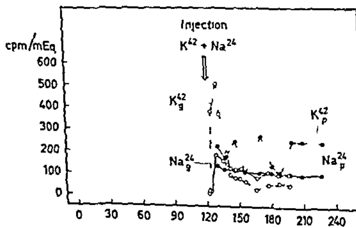
K_g^{42}

Na_g^{24}

K_p^{42}

Minutes

Fig 7 1 b



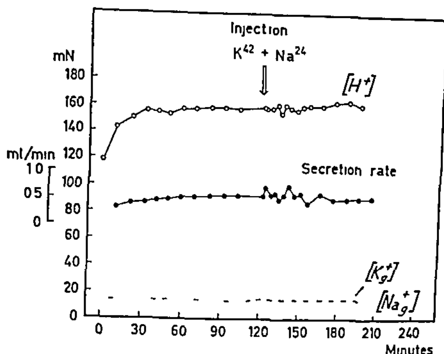


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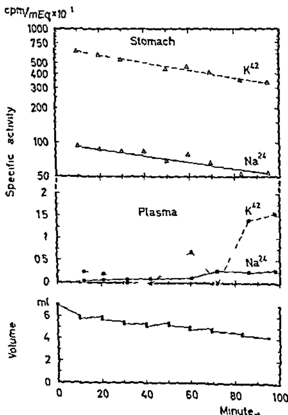


Fig 3 K and Na added to a volume (composition see Table 7 I) instilled in a non secreting stomach Th t p in the volum time curve are due to the removal of small olumes for analy is (Cat 3) It should be noted that although as in Fig the entire stomach contents were removed and then reintroduced during the sampling that this process has been plotted as though requiring no time This seem justified because in this experiment the intragastric radioactivity represents the source and thus is withdrawn from the animal periodically

specific activity of intragastric to plasma potassium falls significantly below unity while the specific activity of *sodium never falls below* that of plasma One explanation for the low potassium value in gastric juice might be that it originates from a compartment of low specific activity

The intracellular concentration of potassium in the gastric mucosa is much higher than the plasma [K+] (MARTIN 1955 DAVENPORT and ALZAMORA 1962) while the plasma concentration is higher than in

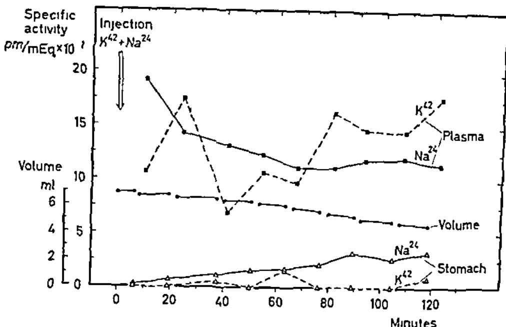


Fig 7 2 The appearance of Na^{24} and K^{42} in a stomach after a single intravenous injection of K^{42} and Na^{24} during secretory arrest with 7 ml solution (composition see Table 7 I) instilled in the stomach Volume in the fig refers to the intragastric volume The sharp decline (left as a discontinuity) in volume between each pair of close points is due to the technique the whole stomach contents were removed a small sample was withdrawn for analysis and the remainder of the gastric fluid was then replaced in the stomach (Cat 4)

(Fig 7 1*a*) It seems most likely that these peaks reflect with some *time lag the earlier peaks in the plasma specific activities resulting* from the injection of K^4 and Na^{24} The width of the peak may be dependent on differences in the transport distance along the crypts In the solution injected the cpm of K^4 and Na^{24} were similar The specific activity of the sodium in the plasma after quasi complete circulatory *mixing therefore became lower than that of* potassium because of the much higher plasma sodium concentration As $[Na^+]$ and $[K^+]$ in the gastric secretion obtained here do not differ very much this should explain the lower specific activity of sodium in gastric secretion

The 8 minute interval between the intravenous injection of the isotopes and the peak of specific activity in the gastric secretion should be a measure of the transit *time of the tracer from* plasma to stomach The approximatively simultaneous appearance of both sodium and potass .m peaks suggest that their transport *mechanisms are similar* or at l ast interrelated Sub equent to the peak value the ratio of the

Net flu of w te f om th crypt into the stomach ca ty

Blood ves el

Flux of K42

C ypt

C ll

E t cellula sp e

F 4 Proposed scheme for the transport of K+ from plasma to the stomach ca ity during ga tric secretion

tracellular concentration of Na (INGRAHAM and VISSCHER 1939 DAVENPORT and ALZAMORA 1962) Thus there is a comparatively large amount of intracellular potassium available which can serve as a diluent for any K^{42} influx A low (compared with plasma) intracellular specific activity might therefore be expected for potassium Secretion of this intracellular potassium into the stomach cavity should therefore give a specific activity in the gastric secretion much lower than that in plasma

To explain the initial peak and the subsequent reduction in the specific activity to a value lower than of plasma it is proposed that duality of the K^{42} pathway exists i e some K^{42} reaches the crypt lumen by a route which does not involve its dilution with the large intracellular pool of K^{39} This pathway might be for example extra cellular or via an intracellular compartment with a low potassium content (Fig 7 4)

Assuming an extracellular pathway the peak of specific activity in the gastric secretion would then be the result of the K^{42} not transported through the parietal cells The experiments were done during steady state gastric secretion The K entering the stomach by the non parietal cell pathway would therefore be continually diluted by a low K^+ fluid originating from the parietal cells (low intracellular specific activity) Thus the specific activity of K in the gastric juice would always be lower than that of the plasma However the time required for the transport of K into the stomach would delay the appearance of the K peak into the gastric juice and the fact that the specific activity in the stomach is at its peak period higher than that of plasma may be merely a consequence of this time lag

TABLE 7 I *Mean values of plasma and stomach contents from the studies of radioactive potassium and sodium movements across the stomach mucosa The mean values represent 5–14 individual samples, usually 10–14*

Cats 3 and 7 were experiments with the isotopes instilled in the non secreting stomach *Cats 4 and 5* were experiments with intravenous injection of the isotopes and the stomach in secretory arrest In the other experiments the isotopes were injected intravenously during gastric secretion

Experiment	Rate of gastric secretion ml/min	Acidity mN	Plasma mN		Gastric contents mN		Plasma cpm		Gastric contents cpm		Quotient of specific activity of: Potassium	Sodium
			Na^+	K^+	Na^+	K^+	Na^{24}	K^{42}	Na^{24}	K^{42}	gastric secretion / plasma	gastric secretion / plasma
Cat 2	0 37	138	145	4 3	13 8	16 9	2438	238	301	437	0 51	1 36
Cat 6	0 56	159	132	3 3	7 9	14 3	2790	130	171	190	0 29	0 94
Cat 8	0 44	158	154	2 7	11 8	12 7	355	24	31	21	0 21	1 18
Cat 10	0 76	159	158	2 6	12 9	10 6	2237	6[illegible]3	207	480	0 21	1 12
Cat 12	0 2[illegible]	136	156	3 1	33 3	11 4	3261	342	6[illegible]6	406	0 44	1 05
Dog Lotta I	0 15	131	142	4 4	25 7	4 9	1324	74	191	54	0 58	0 88
Dog Lotta II	0 [illegible]0	146	148	3 6	8 2	3 7	800	10[illegible]	46	42	0 41	1 20
Dog 1	0 19	102	154	4 4	40 4	3 4	2221	649	622	412	0 67	1 07
Dog 2	0 [illegible]4	136	146	2 8	12 0	2 [illegible]	149[illegible]	216	128	138	0 77	0 99
Dog 3	0 31	133	150	2 9	13 2	2 8	1328	1[illegible]	121	103	0 74	1 06
Cat 3			1[illegible]2	3 8	113 7	10 0						
Cat 4			149	3 3	11[illegible] 1	8 2						
Cat 5			148	3 2	144 2	7 4						
Cat 7			136	4 [illegible]	120 6	9 4						

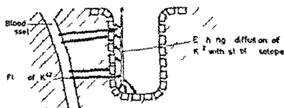


Fig 7 5 Proposed scheme for the tran port of K from plasma to the stomach cavity during secretory arrest

the blood flow in the mucosa is increased and thus larger amounts of radioactive plasma pass by

In these experiments the volume in the stomach was not renewed at the sampling times after removal of a 0 2 ml sample for the analyses the rest of the gastric contents were returned to the stomach Thus a peak such as was found in the secretion experiments (where the stomach was emptied completly at each sampling) would be manifest as a region of much greater slope in the graph relating the specific activity to time In addition the intraga tric volume was rather large and in these cases there was a rather large dilution of any isotope entering which would have the effect of making the determination of such a peak in sodium output more difficult The K output was in any case so low as to be barely significant

The low K+ output seems likely to be due to the large intracellular dilution space available to it (Fig 7 5)

The importance of the crypts for the ion exchange Except for part of the mucus the gastric secretion originates from the crypts in the stom ach wall In cats these crypts are about 1 mm in length (TOLDT 1881) The di tance between the extracellular space and the crypt lumen i of the order 10–50 μ (JOHNSON 1910) According to TOLDT (1881) there are about 75 cryp ts per mm² of the stomach mucosa The average diameter of the crypts (after histological treatment in which shrinking occurs which mu t gi e an underestimate) was about 0 05 mm (TOLDT 1881) The e figures yield a very approximate value of about 12 mm² of internal crypt area per mm² of surface area tangential to the stom ach cavity This calculation suggests that by far the biggest area for diffu ion is in the crypts which would thus be expected to constitute

The specific activity of sodium in the gastric contents never fell below that of plasma It must therefore be concluded that unlike K^{42} the Na^{24} in its passage into the crypt lumen does not become diluted by the addition of its non radioactive isotope Thus the sodium pattern is consistent with a simpler e g a unicompartmental system such as diffusion through the mucosa The intracellular sodium content is much lower than that of potassium and the intracellular compartment is thus a much smaller dilution pool for Na^{24} than for K^{42} The specific activity of sodium emerging from a cell will therefore be much nearer the plasma level than in the case of potassium

After the peak period the specific activity of sodium in the gastric secretion did not fall lower than that in plasma

II K^{42} and Na^{24} injected intravenously and with an isotonic Na/K solution instilled into the non secreting stomach

When comparing the results with the different experimental conditions the question arises as to wether other models fitting the experimental data can be proposed What here has been called diffusion might be influenced by parameters which are difficult to measure such as non acid components It is also quite possible that other factors exert some influence on the movements of the radioactive tracers through the stomach mucosa The electrical potential (i e the forces manifested as the electrical potential) might be different (although this difference is not very big) during secretion and secretory arrest Here a change in electrical potential in the mucosa should however influence sodium and potassium in the same direction and is thus unlikely to account for the result obtained A result here worthy of note is that sodium enters the instilled volume less slowly than potassium during secretory arrest (Fig 7 2) although the ionic mobility of potassium is greater The non secretory conditions do not differ from those at secretion only by virtue of the absence of the water secretion In secretion there is also secretion of H^+ and Cl^- A low pH of the crypts for instance may profoundly affect the permeability properties of the crypt walls Other local conditions resulting from secretion such as changed potential differences across the parietal cell membranes may also be important as regards K (or Na) movements In addition it must be noted that the functional vascular pattern is also much affected in the secretory state During secretion

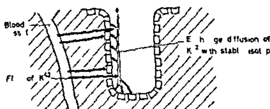


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the predominant transport route from both the extracellular and the intracellular spaces to the stomach cavity

During secretion the ions can be transported *through the crypts* into the stomach cavity with the water flux. In the absence of secretion *transport from crypt to stomach cavity* can occur only by diffusion if there is no mechanical stirring or squeezing.

HEINZ (1951) in an attempt to prove the validity of the glycine instillation method for determining the primary acidity calculated that in a resting stomach glycine diffusion along a crypt 30 μ in diameter and 300 μ long should be 90 % complete in 1½ *minutes*. *This* calculation applied however only to the outer wider part of the *crypt and the real diffusion time should therefore be somewhat* greater. It however seems likely that the time required for diffusion of K^{42} and Na^{24} into the crypts from the stomach cavity is not the rate limiting step as regards their disappearance after instillation.

As it takes less than 8 minutes for the tracer peak to appear in the gastric contents during steady state secretion isotope transport from the plasma to the crypts must take not more than 8 minutes.

In the instillation experiments at secretory arrest with the isotopes given intravenously K^{4} output into the stomach was very slow while there was a much greater and steady increase of Na^{4} *(Fig 7 2)*

Regarding the sodium the plasma specific activity is that after the Na^{24} has already been diluted with a large sodium pool in plasma and in the extracellular or intracellular spaces there is a relatively much smaller diluting pool for Na than for K. Hence it is not unexpected that the Na^{24} activity in stomach is much higher compared with the plasma level than in the case of K. This should also mean *that the actual* K^{4} *output to the stomach should be less*

This fact that sodium increases less slowly than potassium in the instilled intragastric solution may involve *other factors. It has been* found also that sodium apparently moves faster than potassium in other biological tissues. Thus for instance TASAKI, TEORELL and SPYROPOULUS (1961) showed that intracellularly injected Na^{24} passed squid axon membranes faster than K^{42}.

An active process such as expulsion of sodium from the intracellular space as proposed by USSING (see for instance USSING *et al* 1960) could be *utilized to explain a smaller* distribution volume in the gastric mucosa for sodium compared with potassium. The net effect with such a *system on* K^{42} *and* Na^{4} *passing through the mucosa*

would be that potassium would be retained more than sodium similarly as for instance in gel filtration with apparently different distribution volumes for the ions (MARSDEN 1963)

III K^{42} *and* Na^{24} *in the instilled volume during secretory arrest*

These experiments show that diffusion from the stomach is a rather slow process with less than 40 disappearance of the specific activity from the instilled volume in an hour The observation that the K^{42} activity disappears somewhat faster than Na^{24} from the stomach supports the hypothesis that K^{42} equilibrates with a mass of potassium larger than the Na pool available for Na^{24} equilibration This must mean that the K^{42} passes largely into the intracellular space (This is in accordance with the conclusion that potassium to a certain part exchanges with the intracellular space in the cells in the stomach mucosa via the crypts)

General Summary

The aim of the investigation was to study the kinetics of volume secretion and ionic transport rate (H^+ K^+, Na^+ and Cl^-) in the intact stomach of man and dog Especial emphasis has been placed on the [H^+] and [K^+] relationships

Procedure

Human subjects and dogs with gastric fistulae were used A steady gastric secretion rate was induced by the continuous intravenous injection of histamine An antihistamine (chlorpheniramine) was given before the injection of histamine in order to reduce its side effects The bidirectional movements of potassium and sodium were also studied *with the use of radioisotopes* These experiments were performed on dogs and cats mostly under general anaesthesia

Methods of analysis

The methods used are described in the text in connection with the relevant chapters They include the following analyses (1) acidity (2) pH (3) chloride (4) sodium and potassium (5) radioactive sodium and potassium

The relationship between the injection rate of histamine and rate of gastric secretion

The importance of steady state conditions during stimulation is stressed The relationship *was found to be well described by an exponential equation* as proposed by ÖBRINK (1948) It was shown that the antihistamine (chlorpheniramine) had no effect on the gastric secretion Three parameters describing the relationship between the rate of secretion and the dose of histamine were determined and dis

cussed They were the maximal rate of gastric secretion the sensitivity of the mucosa to stimulation and the basal secretion An approximation test for a more rapid estimation of the parameters mentioned is described

In man a basal secretion was always observed but this could not be conclusively demonstrated in dog The initial slope of the rate of volume secretion as a function of the rate of histamine injection was proposed as a measure of the sensitivity of the gastric mucosa to histamine stimulation and thus should give additional information concerning *hyper* or *hypo*secretion of gastric juice

The relationship between the acidity and rate of gastric secretion

The experimental data fitted well the concept of a diffusion like process as the main factor for the acidity regulation The amount of bound acidity was observed to be almost negligible Quantitatively the same relationship was found to be valid in man and dog On theoretical grounds different relationships between acidity and rate of gastric secretion can be expected if different degrees of mixing between the newly formed gastric acid and the preexisting gastric contents occur

The experimental results indicated that normally the mixing is somewhat incomplete *The primary acidity in man was found to be lower than in dog* No evidence was found for other mechanisms that secondarily might lower the acidity in man and the difference in primary acidity in man and dog was considered to be real

An approximation test to evaluate the acidity-secretion rate relationship more rapidly with a graphical method was described and carried out

The permeability coefficient I was proposed as a measure of a possible *hyper* or *hypo*acidity

The relationship between [Cl] and rate of gastric secretion

This relation was found to be quantitatively the same both in man and dog The sum of [K^+] [Na^+] and [H^+] was equal to [Cl^-] [Cl^-] in the primary secretion was calculated with a graphical method and found to be similar in man and dog

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During secretion the intravenously injected K^{42} and Na^{24} were found to exhibit a peak value in the gastric secretion After the peak the specific activity of potassium was found to be considerably lower at the same time as the specific activity of sodium was almost the same as in plasma

When K^{42} and Na^{24} were given intravenously during secretory arrest only part of the sodium activity and almost none of the potassium activity had passed into the stomach after an hour

During secretory arrest with the isotopes instilled in the non secreting stomach the specific activity was found to decline by 23-36 % in an hour with a tendency for potassium to disappear a little faster

To explain these results *it is proposed that potassium in the gastric secretion originates at least partly from the intracellular space in the gastric mucosa*

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Relationship between [Na^+] and rate of gastric secretion

The same quantitative relationship was found in man and dog The data fitted reasonably to a hyperbolic regression equation derived from the diffusion hypothesis The results do not contradict the assumption that there is no sodium in the primary secretion

The relationship between [K^+] and rate of gastric secretion

During a steady rate of gastric secretion [K^+] was observed to be fairly constant A straight line relationship was found to fit the experimental data rather well both in man and dog An essential difference between man and dog was found With increasing rates of gastric secretion [K^+] increased in man In dog [K^+] remained about the same concentration at different secretory rates At very low rates of secretion [K^+] tended to be the same in man and dog The K^+ content in the gastric secretion was concluded to be dependent on the secretory process

The lower primary acidity in man corresponded with a higher [K^+] than in dog, so that the sum of [H^+] and [K^+] in the primary secretion was similar in man and dog It was proposed that K^+ in the gastric secretion was partly of intracellular origin

The effect of histamine on plasma [K^+]

It has been reported that histamine liberates potassium from body tissues (e g MacMillan et al 1956) The importance of these findings were investigated in order to assess their possible significance with regard to the differences in [K^+] in gastric secretion between man and dog Comparison of plasma [K^+] before and during histamine administration (*in vitro* and *in vivo*) showed no significant evidence of increase in plasma [K^+] with histamine administration The conclusion was drawn that [K^+] in gastric secretion depends on some local process in the mucosa

Analysis of the fluxes of K and Na in the stomach

The specific activities of potassium and sodium were measured in plasma and gastric contents during steady gastric secretion and during secretory arrest

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DIFFERENCE BETWEEN ISOLATED NEURONAL AND VASCULAR GLIA WITH RESPECT TO RESPIRATORY ACTIVITY

BY

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Theories in contradiction to the original concept of the neuroglia as a purely supportive tissue in the nervous system were put forward as early as the 19th century. Thus Nansen (1887) postulated that the glial cells are the site of intelligence since their number increases with rising age.

Considerable interest has long been focused on the morphology of the neuroglial cells and the features established 40—50 years ago by light microscopic studies are in fact still mainly valid today.

On the other hand many years elapsed before attempts were made to analyze the function of these cells experimentally, even if certain conclusions had been drawn from studies of pathological material. As late as 10 years ago practically no data were available on this matter. Glees pointed out in 1955 that the observations that would be instrumental in solving the problem of glial function would in all probability be made by the biochemists of the future. Actually it is only during the past five years that the neuroglia and its function has entered into the foreground of experimental research. The conference on the biology of neuroglia held in 1956 was largely responsible for stimulating work that would lead to elucidating the properties of the glial cells. This conference was followed by publication of the monograph *Biology of Neuroglia* (Windle 1958), in which the results already achieved were presented as well as accounts of the methods that had proved most appropriate in various fields.

Two questions that are perhaps of essential importance as far as the glial cells are concerned are their relation to the nerve cells and to the blood vessels. In the latter case interest is focused particularly on their role in the exchange of material between blood and brain.

The present paper comprises a report of the following experimental studies.

1. Separation of glial populations on the micro scale in order to produce a biological model system of the grey matter in the brain.

2. Attempts to characterize the different components of the system biochemically.

3. Attempts to produce specific chemical effects on the components by means of exogenous stimuli.

CHAPTER IV

General Discussion

Summary

References

Theories in contradiction to the original concept of the neuroglia as a purely supportive tissue in the nervous system were put forward as early as the 19th century. Thus Nansen (1887) postulated that the glial cells are the site of intelligence, since their number increases with rising age.

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The biological system consisted of a nerve cell and glia surrounding it as well as of glia surrounding a capillary pre or postcapillary vessel The main interest was concentrated on the activity and reactions of the glial cells The analyses were made on isolated glial cells and nerve cells within the lateral vestibular nucleus of Deiters in the rabbit The samples for analysis comprised approximately 10^{-8} g of tissue since this quantity has earlier proved to be a prerequisite for investigation of material that is morphologically homogeneous Zeuthen s (1953) micro diver technique was used for the metabolic studies

CHAPTER I

HISTORICAL BACKGROUND

Morphological Aspects

Following Virchow s discovery in 1846 of the glial cells as the non neuronal cellular component of nervous tissue the advances in glial research are attributable to numerous workers whose names are by now classical Among them was Holmgren (1901 1914 1917) who made a series of morphological observations on the intimate relation between neuroglial cells and nerve cells He demonstrated the existence of fine intercellular connexions and concluded that the glial cells were implicated in nutrition of the nerve cells

The purely descriptive era ended around 1930 By then — due foremost to the work of Cajal and Hortega — three types of neuroglial cell were distinguished i e 1) *astrocytes* star shaped cells with expansions of varying length 2) *oligodendrocytes* adendritic cells with a small round nucleus and scanty cytoplasm and 3) *microglia* adendritic cells with a rod like nucleus as the main characteristic (Glees 1955)

The astrocytes are larger than the oligodendrocytes and have a bigger lighter less rounded nucleus as well as distinct fibrous expansions In addition the astrocytes have thicker processes (vascular end feet)

Some difficulty is encountered in distinguishing astrocytes from oligodendrocytes in view of the existence of many transitional forms This applies especially in reactive stages It has therefore been conjectured that in certain cases the astrocyte may actually be a reactive form of oligodendrocyte (Ramon Moliner 1958 Koenig Bunge & Bunge 1962)

The astrocytes are found insinuated between the nerve fibres in the white matter of the brain as well as in the borderline layer to the pia mater Their occurrence in the grey matter varies from one region to the next They are perineuronal and or perivascular satellites but not in the same way as the oligodendrocytes since they are attached to the neuron or blood vessel by their processes and not by the cell body Certain astrocytes do however have numerous moss like expansions the whole cell body then lying directly on the vessel wall

The *oligodendrocytes* are most abundant in the white matter of the brain where they are present in closely packed rows between the nerve fibres forming

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neuronal glial cells and axon length in Clarke's column indicating that the nerve cell meets its increased energy requirements by appropriating "auxiliary metabolic units" principally in the form of oligodendrocytes

The astrocytes whose processes cover not only vessels but also neurons and oligodendrocytes appear to act as a structural transport system between blood vessels and nerve cells (De Robertis & Gerschenfeld 1961) Moreover they undergo hypertrophy and hyperplasia after *e g* slight transient ischaemia (Lewis & Swank 1953) and in liver damage (Adams & Foley 1953) This is frequently associated with development of additional vascular connexions and sometimes amitotic cell division (Penfield 1932 Lapham 1962) more often leading to nuclear increase than to cell proliferation

Electron microscopic studies Studies of the nervous system with this technique have shown that there is probably no extracellular space in the brain and that the entire space between nerve cells and vessels is filled by glial cells and their processes

Controversial opinions are expressed regarding identification of the different types of glial cell The majority of workers claim that those cells which have a cytoplasm with low electron density and are in intimate contact with vessels are astrocytes (Farquhar & Hartmann 1957 Maynard Schultz & Pease 1957 Gerschenfeld Wald & Zadunaisky 1959 De Robertis & Gerschenfeld 1961) Others regard them as oligodendrocytes (Luse 1956 Dempsey & Luse 1958 Palay 1958)

It has proved almost impossible to induce extracellular cerebral oedema In experimental oedema of various kinds it is the astrocytes (De Robertis & Gerschenfeld 1961) — or according to the other view the oligodendrocytes — which swell instead The other type of neuroglial cell does not seem to undergo a change with an increase in the water content of the brain but is slightly compressed like the neurons Thus although the astrocytes (oligodendrocytes) are cellular they seem to function as a sort of extracellular space in the brain containing a pool of water electrolytes and nutrients (van Harreveld & Schadé 1959)

Maynard Schultz & Pease (1957) calculated that about 90% of the capillary surface of the brain is overlaid by astrocytes having special contact organs Oligodendrocytes and neurons can also achieve partial contact with the capillaries but lack specialized processes

Tissue cultures of the nervous system have opened up new aspects of the function of the glial cells Both astrocytes and oligodendrocytes show rhythmic pulsatile activity (Lumsden & *Pomerat* 1951 Pomerat 1951 1955 Berg & Kallen 1959) Uptake of substances by pinocytosis has been demonstrated (Klatzo & Miquel 1960) as well as transport through cells Mitotic division

the interfascicular neuroglia Functionally the cells are regarded as the central homologue of the neurilemmal cells of Schwann and have been shown to play a role in formation of the myelin sheath The oligodendrocytes are also found in large numbers in the grey matter here they are perineuronal satellites i e , closely surrounding the nerve cell They may be present as perivascular satellites with the cell body lying directly on the small blood vessel and the expansions entwining the endothelium (Penfield 1932)

The cells of the *microglia* — which derive from the mesoderm and do not belong to the neuroglia proper — are scattered throughout the white and the grey matter with both perineuronal and perivascular locations (Glees 1955)

Krypsin Exner (1952) *made differential counts of the glial nuclei in various* parts of the CNS In the cerebral cortex he found about 60% astrocytes *30% oligodendrocytes and 10%* microglia In the medulla oblongata the oligodendrocytes were far more numerous amounting to over 70% of all glial nuclei

The *morphological relations* between nerve cells and vessels in the CNS have been extensively studied and divergent opinions have been expressed Presumably the view most commonly held is that the nerve cell is separated from the surrounding capillaries by a layer of glial cells (Tschirgi 1958 Gerschenfeld Wald Zadunaisky & De Robertis 1959) *Others have shown that the* nerve cell is in direct contact with the vessel at some site or sites (Maynard *Schultz & Pease 1957 Cammermeyer 1960 a Luse & Harris 1960) The conditions* evidently vary from one region of the brain to the next According to Cammermeyer (1960 b) the oligodendrocytes are arranged chiefly in rows alongside the vessels with large agglomerations at their branchings and around neurons On the basis of this observation he suggested that the cerebral blood flow on the capillary level is controlled by the oligodendrocytes

In recent years much attention has been focused on Holmgren s previously mentioned studies of the relation between glial cells and nerve cells Thus Hess (1958) confirmed the existence of glial penetration into the nerve cell in the form of delicate invaginations *Scheibel & Scheibel (1958) described* contacts between oligodendrocytes and the cell body dendrites and axons *of the neuron It has long been known that the glial cells have the ability to* react to damage (Spielmayer 1922) Reactions of both qualitative and quantitative nature by perineuronal glial cells are seen when increased functional demands are made on nerve cells (Kulenkampff 1952 Kulenkampff & Wustenfeld 1954) as well as after their stimulation (Kuntz & Sulkin 1947 a b) This suggests that the glial cells are involved in nerve cell activity *Friede* & van Houten (1962) found a linear relation between the number of peri

Rubinstein, Klatzo & Miquel (1962) found no difference between the enzyme activity of astrocytes and oligodendrocytes under normal conditions. Experimental brain damage leading to oedema was followed, after a certain latency, by an increase in the enzyme content of the astrocytes specific to each enzyme. No histochemical changes occurred in the oligodendrocytes. Friede (1962) stated that normal astrocytes showed the lowest enzyme activity of all cells in the nervous system. Pathologically reacting astrocytes — apparently of the same type in different parts of the brain — exhibited, on the contrary, a dramatic rise in enzyme content, which in certain cases even surpassed that of the nerve cells. The ability of the astrocytes to undergo adaptive enzyme changes is evidently greater than that known to exist in nerve cells and oligodendrocytes.

An uptake and breakdown of glycogen has been localized to the astrocytes (Oksche 1961). This has proved to be related to the state of activity in the nervous system, indicating that the astrocytes partake in some way in glucose supply.

Quantitative macrochemical analyses

The nervous system has a complex structure, the glial cells being insinuated between fibres and neurons. This makes it impossible to obtain more than a minute specimen of purely glial tissue, containing only a few cells. Some attempts have been made to elucidate the metabolism of the glial cells by means of quantitative macrochemical methods. In these studies advantage has been taken of the variations in cellular composition in different regions of the brain, or use made of agglomerations of glial cells in the form of tumour tissue. Separation has also been attempted. The proportion of the chemical activity of the brain that can be ascribed to the glial cells has then been estimated on the basis of these results and of the histological features.

Elliott & Heller (1957) compared the oxygen consumption of the corpus callosum, which has a high content of glial cells, with that of the cerebral cortex and cerebellar cortex. They calculated that the glial cells were responsible for 30 % of the respiration in the cerebral cortex, but that the glial cells of the corpus callosum had a greater oxygen consumption per cell than the nerve cells of the cerebellum.

Abood, Gerard, Banks & Tschirgi (1952) showed that the enzyme activity per unit of fat free dry weight was usually 50 % higher in the grey matter than in the white. Dixon (1953) found that the outermost layer of the cerebral cortex, which has a sparsity of nerve cells, was more active in anaerobic glycolysis than the deeper layers, which have an abundance of these cells. This finding is compatible with the observations in glial tumours that have also

has been observed in glial cells but not in nerve cells. The contribution of glial cells to myelination of nerve fibres is another factor that has been studied in tissue cultures (Hild 1959, Murray 1959).

Electrophysiological aspects. Hitherto little attention has been devoted to this question. Svaetichin *et al.* (1961) concluded on the basis of electrophysiological studies of the nerve cells and supporting cells of the retina that the excitability of the neuron is dependent on the state of these glial satellites. Hild & Tasaki (1962) investigated the electric response of glial cells in tissue cultures. They found the resting potential to lie at 50—70 mV. Electric stimulation produced a reduction in the resting potential which was regained only after several seconds. This slow glial response could also be demonstrated *in vivo*. The authors concluded that a long continuous activity of neurons can alter the state of the surrounding glial cells.

Biochemical Aspects

During the past 10 years or so interest has been focused on the biochemistry of the nervous system. As a result we have gained information about metabolic activities of various kinds in the brain and some of its chemical characteristics have been established. Actually there are many chemical similarities between homogenates of the brain and e.g. liver.

Various methods have been used in biochemical analyses of the CNS and its components.

Histochemistry

The methods are based on the use of certain staining procedures — many of them long established ones — to localize various chemical components, primarily enzymes, in histological specimens.

Early work on the nervous system showed lack of enzyme activity in the glial cells (Marinesco 1919, Rutenburg, Wolman & Seligman 1953). Shimizu & Morikawa (1957) found no succinic dehydrogenase activity in the white matter whereas it was demonstrable in the grey matter both in nerve cells and in neuropil. Friede (1959) observed succinic dehydrogenase activity in glial cells during active myelination but not in adult glia. Wolfgram & Rose (1959) using refined techniques demonstrated several dehydrogenases in all types of glial cell. Thomas & Pearse (1961) reported the glia to have a low content of oxidative enzymes with the exception of glycerol-1-phosphate dehydrogenase which was abundant, whereas only a small amount was present in the nerve cells.

Lowry *et al* 1956) Lowry found that nerve cells had lower activity than brain tissue on the average as far as the important energy transforming systems are concerned a conclusion that he had already drawn from studies of the highly cellular layers of Ammon s horn (Lowry *et al* 1954) The activity of the nerve cells was particularly evident in the case of phosphoglucoisomerase lactic dehydrogenase and glutamic dehydrogenase Lowry *et al* also observed a striking contrast between the enzyme content of nerve cells and that of ganglion cell capsules consisting of glial cells The latter had extremely little hexokinase malic dehydrogenase transaminase and glutamic dehydrogenase whereas their content of phosphoglucoisomerase lactic dehydrogenase and isocitric dehydrogenase was on a par with that of nerve cells In addition they had relatively abundant glucose 6 phosphate and 6 phosphogluconate dehydrogenases

Working with micro methods Hydén and co workers studied isolated nerve cells and glial cells with regard to mass organic material nucleic acid and enzymes Quantitatively considerable differences were noted between the nerve cell and the glial cells directly surrounding it in several respects The glia contained only one tenth the amount of RNA determined on the same dry weight basis as its neuron (Hydén & Pigon 1960 Egyházi & Hydén 1961) Qualitative differences were observed in the RNA of the giant nerve cells and their glia in the lateral vestibular nucleus of Deiters This was reflected in higher guanine and lower cytosine values in the nerve cell and vice versa in the glial cell as determined by Edstrom s micro electrophoretic method

In a study of the cytochrome oxidase and succinoxidase activity in isolated spinal ganglion cells and their glial satellites Hydén Løvtrup & Pigon (1958) found considerably higher values in the latter than in the ganglion cells expressed both per volume and per dry weight This also applied to Deiters nerve cells and their glia (Hydén & Pigon 1960) Glutamate was on the contrary oxidized at a far higher rate by the nerve cells and α ketoglutarate about as rapidly by both nerve cells and glial cells (Hamberger 1961)

The glial ATPases displayed higher activity than that of the nerve cells in Deiters nucleus surrounded by them Furthermore the two types of cell differed with respect both to pH maxima and ion requirements (Cummins & Hydén 1962)

Studies chiefly on the lateral vestibular nucleus suggest a metabolic relation between the nerve cell and its surrounding glia After intermittent vestibular stimulation the RNA and protein content of the nerve cells in Deiters nucleus increased significantly as did the respiratory enzyme activities In the glial cells a decrease in the corresponding values was recorded (Hyden & Pigon 1960) In those nerve cells whose enzyme activity was raised anaerobic glyco

proved to have a high glycolytic capacity under anaerobic conditions Korey & Orchen (1959) recovered a fraction containing non neuronal cells chiefly glial cells by means of separation and sedimentation The respiration per glial cell amounted to 10^{-5} µl O /hour The ratio of nerve cell to glial cell respiration in the cerebral cortex was calculated to be about 1 1

Victor & Wolf (1937) and Elliott & Heller (1957) found oligodendrogliomas to exhibit considerably greater oxygen uptake than astrocytomas Allen (1957) demonstrated that the well differentiated astrocytomas had a cyto chrome oxidase activity that was even lower than that of the white matter In the well differentiated oligodendrogliomas it was on the contrary, many times greater amounting to about two thirds of that in the cerebral cortex Another characteristic of the glial cells that has been studied in tumour tissue is their high pseudocholinesterase content (Bulbring Philpot & Bosanquet 1953 Cavanagh Thompson & Webster 1954) The aforementioned authors have intimated that the difference between astrocytomas and oligodendrogliomas may reflect differences between the respective normal cells

Quantitative histo and cytochemistry

Methods of this type use exceedingly small amounts of biological material to permit direct correlation between chemical and histological observations With the chemical analytic technique elaborated by Linderstrom Lang (1939) quantitative data can be obtained from biological samples with a dry weight as low as 0 2 µg Pope (1952 1958) and Pope *et al* (1956) studied the enzyme distribution in *e g* the cell layers of the cerebral cortex They stated that the respiratory enzymes were localized mainly to the cell body and dendrites of the neuron whereas the glial cells had a proteolytic activity of the same order of magnitude as the nerve cells

Lowry has devised methods to comprise determinations of a large number of enzymes and has achieved a sensitivity that allows analyses to be made of material much smaller than a single glial cell (Lowry *et al* 1961) The deter minations are made on freeze dried material using chiefly fluorimetric tech niques Robins & Smith (1953) analyzed oxidative and glycolytic enzymes in the cerebellum With few exceptions particularly cholinesterase the molecular layer showed higher activity than the granular layer this in its turn was more active than the white matter Robins Smith Eydt & McCaman (1956) showed that in the cerebral cortex the activity of several enzymes was on a high level in the outer layers and fell successively towards the white matter

The possibility of analyzing individual cells implied a remarkable advance in the quantitative histochemistry of the nervous system (Lowry 1957 a b

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lysis was depressed whereas it was significantly augmented in the glial cells (Hamberger & Hydén 1963) Changes in RNA in the glia and nuclei of the neurons were also observed in rats learning to perform a complicated motor pattern (Hydén & Lgyházi 1962)

When enhanced synthesis of protein and RNA was induced by chemical agents the nerve cell responded by a significant increase in both cases In the glial cell the RNA content fell significantly The RNA base composition of the nerve cell and its glia exhibited inverse changes with respect to guanine and cytosine values (Egyhazi & Hyden 1961 Hyden & Egyházi 1962)

Giacobini (1956 1957) used the micro diver technique for analysis of the cholinesterase of the nervous system of cells and parts of cells as well for determination of the carbonic anhydrase of nerve cells and glial cells (Giaco bini 1962) He found the latter enzyme to be localized to a high degree to the glial cells

In human pathology glial cell changes are known to be phenomena asso ciated with vascular and inflammatory disturbances among others and are generally preceded by severe damage to the nerve cells In a study of paralysis agitans (Parkinson s disease) biopsy specimens from the globus pallidus were analyzed by microchemical methods (Gomirato & Hyden 1963) In early sta ges qualitative changes in the RNA of the glial cells were present but when the disease was more advanced the changes in the nerve cells were more prominent

CHAPTER II

MATERIAL AND METHODS

Microdissection

Albino rabbits of both sexes were used their weight ranged from 1 [illegible] to 1 8 kg For 8—10 days before the experiments they were kept under uniform conditions with food and water *ad libitum*

The material consisted both of untreated rabbits (controls) and of rabbits exposed to stimulation of various types The experimental conditions in the latter animals are described in Chapter III

The animal was given an air embolus (15 ml of air injected into the ear vein) and became unconscious within a few seconds The carotid vessels were cut to drain the blood from the body This procedure was used to ensure minimum blood filling of the brain The roof of the skull was opened and the brain removed Slices of the part of the medulla oblongata to be studied approximately 2 mm thick were cut free hand About 2 minutes after the animal s death these slices were placed in ice cold 0 [illegible] *M* sucrose solution containing 0 001—0 00[illegible] *M* methylene blue

The nerve cells were faintly stained by the weak methylene blue solution since the dye is taken up only by the synaptic knobs as demonstrated by silver staining of nerve cells (Hyden 1959) The structure of the lateral vestibular nucleus was therefore distinct The addition of methylene blue during the preparation had no influence on the enzyme activity determined

The Lateral Vestibular Nucleus of Deiters

This term is used to denote the part of the vestibular complex in the rhombencephalon that is characterized by the presence of Deiters giant nerve cells In addition to small cells of various types (Brodal & Pompeiano 19[illegible]) The medium sized usually spindle shaped nerve cells are collected in a small *group* in its lateral part In the *rostral* medial part of the nucleus the giant cells are comparatively sparse and are mixed with small nerve cells The giant cells are most numerous and largest in the caudal part of the nucleus where the admixture of small cells is *inappreciable* The vascular supply to the nucleus is relatively abundant (Craigie 1938)

The efferent fibres of Deiters *nucleus* are relayed primarily to the *cervical* thoracic and lumbosacral parts of the spinal *cord* via the homolateral vestibular spinal tract It receives afferent fibres chiefly from the spinal *cord* labyrinth cerebellum and reticular

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Fig 2 Nerve cell (right) and neuronal glia (left) after separation × 200

When the nerve cell was dissected out the glia surrounding the cell body was adherent to it By gentle manipulation in the drop the nerve cell was freed from the glial cells which formed a clump (Fig 2) This clump was shaped into a sphere and trimmed down to approximately the same volume as that of the nerve cell The volume was determined with a micrometer eyepiece After some experience the volume of the glial clump in relation to the nerve cell could be estimated by visual inspection only An account of determination of the volume as a source of error in the method is given on page 28

The glial clump dissected out in this way is denoted in the following as the *neuronal glia*

Capillary glia

To isolate the capillary glia iris scissors were used to cut out a thin flake of tissue about 500 × 400 × 200 μ from which the nerve cells had been picked out (Fig 3) The sample was examined under the microscope to check that no tissue beyond the lateral vestibular nucleus was included Actually there is a good margin since the part of the nucleus in which the giant nerve cells are mainly present measures about 0 6—0 8 mm in the rostral caudal direction (Meessen & Olszewski 1949)

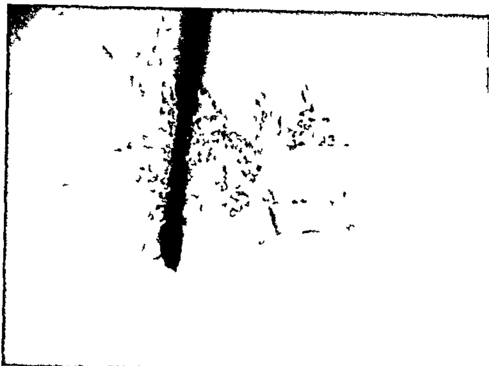


Fig 1 Nerve cell and neuronal glia during separation with the dissecting needle × 200

formation The function of the nucleus is considered to be highly uniform even if the afferent connexions are not the same in all cells However that part of the nucleus used as material in the present investigation is as far as is known both histologically and physiologically homogeneous (Brodal Pompeiano & Walberg 1962)

After about 1 minute in the staining fluid the sections were transferred to isotonic sucrose solution and were examined at 32 or 50 times magnification under a Zeiss stereomicroscope with the light falling obliquely on the slice

Neuronal glia

The neuronal glia was dissected out with the method of Hydén (1959)

The nerve cells were lifted up from the tissue slice and placed in a small drop of incubation medium with a stainless steel needle[1] 30 μ thick mounted in a drawn glass tube (Fig 1) Thin glass needles were also tested for this purpose Although they were found to be less practicable they afforded a possibility of comparing the results of dissecting out the cells in different ways No difference in enzyme activity was demonstrable

[1] The stainless steel wire wa supplied by AB Kanthal Hallstahammar Sweden



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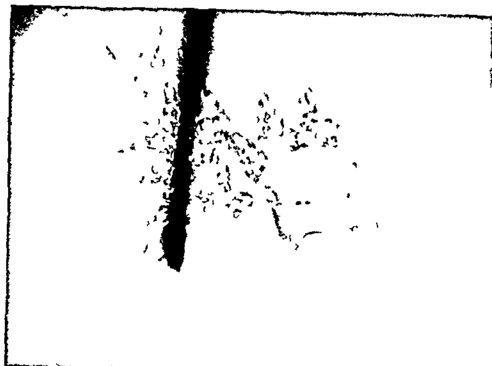


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Fig 5 Thin vessel with adherent capillary glia × 00

The tissue sample was transferred to a drop of incubation medium and divided into small pieces with the help of the aforementioned micro-needles It was then observed that a relatively thick vessel trunk (40–70 μ) ran through most of the pieces The whole vessel could easily be freed from practically all nervous tissue leaving a trunk with branches down to 6–8 μ in diameter However round the thinnest vessels — particularly at their branchings but also at other sites — a little glia remained (Fig 4) it proved to be considerably more adherent to the vessel than the main part of the tissue mass (Fig 5) The nerve cells were visible all the time and resembled transparent raisins in a cake Once the vessel had been separated from the nervous tissue no nerve cell was directly adherent to a capillary nor to the glia still attached to the vessel The glial clumps could be completely freed from the vessel by cautious dissection (Fig 6) They were then formed into spheres in the same way as the neuronal glia their volume in relation to that of the nerve cells was estimated in the same way

The glial clump dissected out in this way is denoted in the following as the *capillary glia*



Fig 3 Flake of tissue from the lateral vestibular nucleus Vital staining of vessels and neurons × 75



Fig 4 Vessel with capillary glia surrounding the thinnest branches × 150

the mean values of less than 5% with a low standard deviation Consequently the activity relations calculated in the present study on a volume basis are also valid on a dry weight basis

Morphological Features of Glial Material

A series of dissected out glial clumps were fixed and stained for identification of their morphological features The clumps were transferred with an agar coated micro pipette from the drop of incubation medium to a glass slide and allowed to dry After staining the number of glial nuclei in each clump was counted There were generally 7–8 in both neuronal and capillary glia although 5 6 9 or 10 nuclei were occasionally present

The degree of purity of the glial samples is an important factor In silver stained histological sections the glia surrounding both the nerve cells and the capillaries is seen to contain a fine network of nerve fibres chiefly thin axon terminals The following calculation was made to ascertain how large a proportion of the volume of the glial clump is represented by these fibres

The volume of the glial clump is approximately 80 000 μ^3 For the sake of simplicity the dimensions are assumed to be 50×50×30 μ with a surface of 2500 μ bordering on the nerve cell Directly beside the nerve cell the fibres are extremely thin their mean diameter is stated by Horstmann & Meves (1959) to be 0 μ According to David (1957) there are 16 synaptic knobs per 100 μ of nerve cell area in the giant cells of Deiters Consequently about 400 small fibres extend into the region closest to the nerve cell here calculated to be at a distance of 15 μ from the surface This implies that their volume is approximately 400 μ^3 or between 1 and 2% of half the glial volume Further from the nerve cells surface the fibres become fewer and coarser i e about 5 per 100 μ^2 and about 1 μ in diameter representing a volume of slightly less than 2000 μ^3 or approximately 5% of half the glial volume

Computing the relative area of the fibres on electron micrographs from different parts of the grey matter published by various authors I have found the mean to be about 5%

On the basis of these figures it can be estimated that the nerve fibres are responsible for about 5% of the total volume of the glial clump

The amount of organic dry substance per μ^3 in the axon has been shown to be about 0 08 $\mu\mu$g (Engstrom & Luthy 1950) and 0 06 $\mu\mu$g (Hydén 1955) whereas the corresponding figure in the glia is 0 0 $\mu\mu$g Consequently the percentage contribution of the fibres is lower from the point of view of organic cell material than from that of volume

The thin nerve fibres have a low mitochondrial content Mitochondria are



Fig 6 Capillary glia during separation from vessel × [illegible]00

Blood vessels

In some experiments the oxygen consumption of the thin vessels freed from glia was studied The rabbits were anaesthetized with Isomyl sodium® (sodium ethyl isoamyl barbiturate) after which the vascular system was perfused *via the heart with physiologic saline to remove the blood* The relative quantity of endothelial cells in the different experiments was determined by measuring the total length of *vessels of different diameter*

Determination of Dry Weight

Hydén (1959) showed that the dry weight per volume unit of the glial cells surrounding Deiters giant nerve cells is 0 20 g cm³ *i e* a value close to that of the nerve cells

A comparison between the dry weight per volume unit of the neuronal and *the capillary glia was made in freeze dried sections by means of X ray micro* radiography at 8—10 Å (Hydén 1959) The dry weight was evaluated with a *scanning microdensitometer (Hydén & Larsson 1956)* The weight of each sample represents the mean of a large number of measuring points A comparison between the mass per volume unit in the glia directly surrounding nerve cells and that round vessels of varying diameter showed a difference in

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Fig Nerve cell (right) and glial clump (left) Diver tip inserted in incubation medium for filling × 200

Morphological Features During Diver Experiment

In order to study the morphological features of the nerve cells and glial cells under the conditions of determinations with the micro diver technique — made directly after dissection — filled micro divers were incubated under a photo micro cope for 24 hours at 37 C (A diver experiment is discontinued 3–4 hours after the animal s death) After this time no morphological changes were observed in either type of cell at 0 3 μ maximal resolving power as far as membranes and volume were concerned Nor were any changes demonstrable after a further 20 hours

Analytical Methods

The oxygen uptake of nerve cells and glial cells was determined by the micro diver technique (Zeuthen 1953 Zajicek & Zeuthen 1956 1961) as applied to nervous tissue by Hydén & Pigon (1960)

Divers

The divers were made of thin walled Pyrex glass capillaries about 1 mm in diameter The capillary was fastened in a glass handle with de Khotinsky

present in the synaptic knobs but as demonstrated earlier, they remain on the surface of the isolated nerve cell and are not included in the glial sample (Hydén 1959) The glial cells — particularly the oligodendrocytes — have on the contrary a relatively large number of mitochondria (De Robertis & Gerschenfeld 1961)

Since in the present study determinations are made of respiratory enzymes that are bound to the mitochondria the activity recorded must reflect almost exclusively the respiration of the glial cells

An attempt was made in the following way to estimate the distribution of the two classical types of neuroglia *i e*, oligodendrocytes and astrocytes in the neuronal and capillary glia

Dissected out fixed glial clumps were stained with toluidine blue cresyl violet Cajal s gold sublimate and Hortega silver carbonate respectively with certain modifications In view of the difficulties associated with the gold and silver impregnation methods as well as the special preparation of the tissue the cytoplasmic features of the glial cells could not be brought out distinctly enough Since classification of glial cells is based partly on these features some authors do not regard the appearance of the nucleus alone to be an entirely reliable criterion The appearance of the nucleus is however characteristic and has often been used as the sole criterion in determining the type of cell (Krypsin Exner 1952 Cammermeyer 1960 a b Smart & Leblond 1961 among others)

The oligodendrocyte has a small round dark nucleus containing large clumps of chromatin The astrocyte has a larger oval or irregular nucleus with a light chromatin pattern (Penfield 1932) A number of transitional forms exist The microglia which are relatively sparse were not studied in any detail in the present investigation

On the basis of the aforegoing criteria the neuronal glia was found to consist of about 90% oligodendrocytes and 10% astrocytes and the capillary glia of 70% oligodendrocytes and 30% astrocytes These calculations were made on several series of glial clumps totally about 100 clumps stained with the techniques just listed

For comparison the same staining procedures were applied to histological sections The perfusion technique of Koenig Groat & Windle (1944) was used to prepare the vascular system The vessels were demonstrated with a mixture of 3% gelatin solution and Indian ink The glial cells can obviously be differentiated with greater certainty in sections Practically all glial cells around Deiters giant nerve cells were of oligodendrocyte type Hydén & Pigon (1960) have given their incidence as 90% Although astrocytes were present beside the capillaries the oligodendrocytes predominated here as well



Fig ". Nerve cell (right) and glial clump (left). Diver tip inserted in incubation medium for filling × 00

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Fig 8 Glial clump entering the diver × 200

cement so that the diver formed the tip of a fine braking pipette The diver tip was then introduced into the drop of incubation medium containing a nerve cell or neuronal or capillary glia Under the stereomicroscope the diver was filled with one of these samples in a little incubation medium as shown in Figures 7 and 8 after which the tip was sealed with beeswax heated to melting point (about 60° C) The diver was then detached from its handle and adjusted to flotation equilibrium in *0 1 M* phosphate buffer pH 7 4 This implies that an air bubble in the ampoule (the thickest central part of *the diver) was bordered by the incubation medium and the biological material* in the sealed end and by the phosphate buffer in the open end (Figs 9 and 10) The size of the air bubble was such that the diver was just able to remain afloat in the phosphate buffer The diver was then ready to be transferred to the water bath — A trained worker can fill a diver and transfer it to the water bath within about 20 minutes of the animal s death

Each diver was about 15 mm long had a weight of 0 2—0 6 mg contained about 0 3 μl of incubation medium and an air bubble with a volume of 0 1—0 3 μl The ratio of the weight of the glass used for the diver to the volume of air required to keep it afloat in the medium was determined for every sample of glass tubing

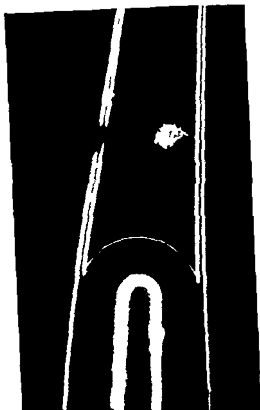


Fig 9



Fig 10

Fig 9 Position of glial clump in the diver At bottom air bubble × 45

Fig 10 The whole diver The dotted rectangle represents the part shown in Fig 9 × 5

Water bath

The divers were transferred to flotation vessels These were immersed in a thermostat regulated water bath heated by two 250 W lamps The temperature variations in the bath were measured by NTC resistors connected in a bridge during an observation period of several hours they remained below ±0 005 C All measurements were made at 37 C Four divers were run simultaneously

Flotation medium

The flotation vessels were filled with 10 ml of flotation medium This consisted of the same phosphate buffer as that used in adjusting the divers

In analogy with the Warburg method and the standard diver 0 1 N NaOH was used in an earlier study to absorb the carbon dioxide (Hamberger 1961) It has subsequently been found that the solubility of carbon dioxide in the phosphate buffer is sufficiently great for the quantities relevant in the diver determinations

Enzyme assays

The enzymic parameters measured in nerve cells and glial cells were the aerobic oxidation of cytochrome *c* (cytochrome oxidase), succinate, α ketoglutarate and glutamate

Cytochrome oxidase Incubation medium Na HPO_4–KH PO_4 buffer pH 7 4 37 5 mM cytochrome *c* 8 6×10⁻ mM Na ascorbate 12 5 mM $AlCl_3$ 0 5 mM (Slater 1949 a Potter 1957) The concentrations refer to the final mixture in the diver All reagents were mixed directly before the experiment The Na ascorbate was prepared freshly before final mixing of the medium by neutralizing a solution of ascorbic acid with 0 1 M NaOH

A complication in the cytochrome oxidase determinations is the autoxidation *i e* oxidation of the reduced cytochrome *c* in the absence of tissue (Slater 1949 a Potter 1957 Hyden Lovtrup & Pigon 1958) This is believed to be caused by insufficient purity of the reagents The pH of the incubation medium is also of importance Schneider & Potter (1943) determined the oxidation in the blank by concurrent determination with different amounts of enzyme and extrapolation to zero amount of enzyme This method could not be employed in the diver experiments in view of the differences in activity in morphologically identical nerve cells, as well as in clumps of glia Instead a diver containing incubation medium but no enzyme represented the autoxidation blank whose activity was subtracted from the values recorded in the other divers in the same run This procedure has been used in earlier determinations of cytochrome oxidase activity in nerve cells and glia by the micro diver technique (Hydén Lovtrup & Pigon 1958 Hydén & Pigon 1960)

Succinate oxidation Incubation medium Na_2HPO_4–KH PO_4 buffer pH 7 4 37 5 mM cytochrome *c* 8 6×10⁻ mM Na succinate 25 mM $AlCl_3$ 0 5 mM $MgCl$ 0 5 mM (Slater 1949 b Potter 1957) The rate of oxygen uptake with succinate was found to be the same in this medium as in that used for determination of α ketoglutarate and glutamate (*vide infra*)

The influence of succinate concentration on the oxygen uptake was investigated (Fig 11) Parallel curves for activity and substrate concentration were obtained in nerve cells and in glial cells and oxygen uptake was maximal

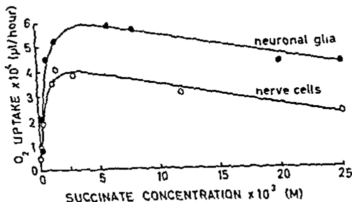


Fig 11 Succinate oxidation by Deiters nerve cells and neuronal glia correlation between enzyme activity and substrate concentration

at a slightly lower concentration than that used here Slater (1949 b) working with heart-muscle particles also found the enzyme activity to be inhibited by a high succinate concentration He reported the optimal concentration to be about 0 0? M

α Ketoglutarate and glutamate oxidation Incubation media $MgSO_4$ 6 ? mM KCl 25 mM Na_2HPO_4-KH_2PO_4 buffer pH 7 4 13 3 mM cytochrome c $2 \times 10^{-?}$ mM ATP 1 mM sucrose 175 mM and Na α ketoglutarate 13 mM or Na l glutamate 15 mM (Christie Judah & Rees 1953 Hamberger 1961 Løvtrup & Svennerholm 1963)

In the case of succinate α ketoglutarate and glutamate oxidation the autoxidation was negligible

As far as isolated cells were concerned the influence of certain variables in the incubation media was investigated with the following results The substrate concentration was high enough to induce maximal oxygen uptake The activity was slightly reduced by omission of cytochrome *c* Addition of DPN or calcium ions had no appreciable effect — A complete accurate analysis of the influence of changes in the incubation media was considered difficult because of the large variations in enzyme activity inherent in biological material

Measurements and calculation of results

When the divers had been transferred to the flotation vessels and immersed in the water bath temperature equilibrium was reached after about 30 minutes Manometric readings of the *equilibration* pressure were then made every 15—20 minutes for 2—3 hours The pressure was plotted against the time

and a linear relation was found to be present during the first 3—4 hours and often for a longer period

The enzyme activity is expressed as the oxygen uptake in $\mu l \times 10^{-4}$/hour per nerve cell or per corresponding volume of glia The O uptake was calculated according to Zeuthen (1953)

Error of the Method

The chief factors on which the error of the method is dependent are

1 The accuracy of the micro diver method
2 The accuracy of determination of the volume of the glial sample

1 — In theoretical calculations Zajicek & Zeuthen (1961) found the error of the diver method to be $\pm 5\%$ in determination of carbon dioxide production of the order of magnitude of 10^{-4} μl/hour Experimentally they achieved the same accuracy by filling three divers with an equal quantity of human plasma cholinesterase A suspension of isolated mitochondria with succinate as substrate was used in the present investigation to determine the accuracy in measurements of oxygen uptake Four divers were filled with an equal quantity of suspension The oxygen consumption was 16 8 15 2 15 1 and 18 7×10^{-4} μl/hour *i e* a standard error of approximately $\pm 10\%$

2 — As stated earlier the axes of the glial clumps were initially determined with a micrometer eyepiece The volume was calculated as an ellipsoid The routine method of calculation subsequently used was based on visual inspection only That it was in fact sufficiently accurate can be inferred from the results of investigations by various workers who have applied it The standard deviation was not larger for the glia than for the nerve cells in the present study it was even slightly smaller

Statistical Analyses

The results of the respiratory measurements are recorded in Tables I—VI The mean standard error of the mean (S E M) and the number of samples in each series (n) *i e* the number of animals are given in all but Table V

In the controls the significance of the difference between nerve cell and neuronal glia as well as between neuronal and capillary glia was tested by customary statistical methods (Hald 1952)

In the animals stimulated in various ways the t test was used to test the significance of the difference between these samples and samples of corresponding cell types in the controls

A biologically significant difference was considered to be present when p was less than 0 05

CHAPTEP III

RESULTS

The neuron and its glia i e the neuronal glia is the unit of the lateral vestibular nucleus of Deiters previously studied by the micro diver technique (Hydén & Pigon 1960 Hyden & Lange 1962 Hamberger & Hydén 1963) The grey matter of the brain consists of neurons glia and blood vessels the glia being responsible for a large proportion of the volume The main object of the present investigation was to obtain data on the capillary glia and to study a model system with three components nerve cell neuronal glia and capillary glia The fundamental problem was to answer the following questions Is the metabolism of the glia although susceptible to the influence of various factors uniform throughout its mass and therefore demonstrable by micro analyses of the neuronal glia? Or does its metabolism exhibit a well differentiated pattern indicative of a functional distribution?

The first step was therefore to study the metabolism — in terms of the respiratory enzyme activity — under normal conditions In subsequent experiments with stimulation of various kinds controls were included in every series as a check that no changes had occurred in the experimental conditions

The mean values for nerve cell neuronal glia and capillary glia are given in all tables

TABLE I

Enzyme a tivity i nerve cells neuronal and cap llary glia of Deiters *nu leu controls*
Enzyme act vity xpressed as 10^{-4} $\mu l\ O_2$/hour *Mean values* ±S E M

	NERVE CELL	n	NEURONAL GLIA	n	CAPILLARY GLIA	n
CYTOCHROME OXIDASE	42 06[x]	16	115 08[x]	17	116 11	10
SUCCINATE OXIDATION	22 03[x]	17	42 05	9	69 07	9
α KETOGLUTARATE OXIDATION	22 04	9	21 02	12	40 07	13
GLUTAMATE OXIDATION	22+03[xx]	21	11 02	9	16 04	10

x From Hydén & Pigon 1960
xx From Hamberger 1961

and a linear relation was found to be present during the first 3–4 hours and often for a longer period

The enzyme activity is expressed as the oxygen uptake in $\mu l \times 10^{-4}$/hour per nerve cell or per corresponding volume of glia. The O_2 uptake was calculated according to Zeuthen (1953).

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1. The accuracy of the micro diver method.
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2. – As stated earlier the axes of the glial clumps were initially determined with a micrometer eyepiece. The volume was calculated as an ellipsoid. The routine method of calculation subsequently used was based on visual inspection only. That it was in fact sufficiently accurate can be inferred from the results of investigations by various workers who have applied it. The standard deviation was not larger for the glia than for the nerve cells in the present study; it was even slightly smaller.

Statistical Analyses

The results of the respiratory measurements are recorded in Tables I–VI. The mean, standard error of the mean (S.E.M.) and the number of samples in each series (n), i.e. the number of animals, are given in all but Table V.

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CHAPTER III

RESULTS

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The first step was therefore to study the metabolism — in terms of the respiratory enzyme activity – under normal conditions In subsequent experi ments with stimulation of various kinds controls were included in every series as a check that no changes had occurred in the experimental conditions

The mean values for nerve cell neuronal glia and capillary glia are given in all tables

TABLE I

Enzyme activity in nerve cells neuronal and capillary glia of Deiters nucleus controls
Enzyme activity expressed as 10^{-4} µl O /hour Mean values ±S E M

	NERVE CELL	n	NEURONAL GLIA	n	CAPILLARY GLIA	n
CYTOCHROME OXIDASE	42 06 [x]	16	115 08 [x]	17	116 11	10
SUCCINATE OXIDATION	22 03 [x]	17	42 05	9	69 07	9
α KETOGLUTARATE OXIDATION	22 04	9	21 02	12	40 07	13
GLUTAMATE OXIDATION	22 03 [xx]	21	11 0.2	9	16 04	10

x From Hydén & Pigon 1960
xx From Hamberger 1961

CONTROLS

The values listed in Table I are based on analyses of samples from 49 control animals

Cytochrome Oxidase

Cytochrome oxidase the catalyst of electron transfer from cytochrome c to oxygen is the terminal enzyme of the respiratory chain It is seen in Table I that in the isolated nerve cell the absolute value for this activity 4 2 $\times 10^{-4}$ μl O_2 per hour was higher than for any of the other oxidative activities investigated The cytochrome oxidase activity in the neuronal and capillary glia was almost three times as great as that in the nerve cell The activity was practically the same in the capillary and in the neuronal glia The variations between observations in each group after correction for autoxidation were no greater than in other parameters studied

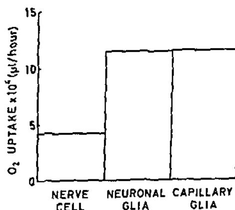


Fig 12 Cytochrome oxidase in controls

Succinate Oxidation

As in the case of cytochrome oxidase succinate oxidation was lowest in the nerve cell It is interesting to note that a difference was found between neuronal and capillary glia the activity of the latter being considerably higher There seemed to be an activity gradient from vessel to neuron

In a preliminary experimental series it was found that the difference between the two types of glia was less marked if 10 ml of Indian ink were injected intravenously a few minutes before the animals were killed for better visualization of the vessels The mean value of succinate oxidation in the

capillary glia was then 0.9×10^{-4} µl O per hour whereas the corresponding value in the neuronal glia (4.2×10^{-4} µl) as well as in the nerve cell (2.2×10^{-4} µl) was the same as in the latter series The decrease in activity in the capillary glia may have been due to a toxic effect of the Indian ink primarily localized to the immediate vicinity of the vessels

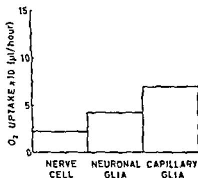


Fig 13 Succinate oxidation in controls

α Ketoglutarate Oxidation

This activity was as high in the nerve cell as in the neuronal glia — a unique circumstance among the oxidative mechanisms studied here The activity of the capillary glia was about 100% higher than that of the neuronal glia thus an observation similar to that in succinate oxidation

The improvements in the method described in the previous chapter gave as a rule mean values that were slightly higher than those reported earlier

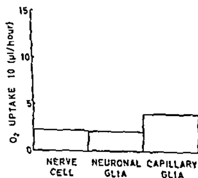


Fig 14 α Ketoglutarate oxidation in controls

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Cytochrome oxidase the catalyst of electron transfer from cytochrome *c* to oxygen is the terminal enzyme of the respiratory chain It is seen in Table I that in the isolated nerve cell the absolute value for this activity $4\ 2 \times 10^{-4}$ $\mu l\ O_2$ per hour was higher than for any of the other oxidative activities investigated The cytochrome oxidase activity in the neuronal and capillary *glia was almost three times as great as that in the nerve cell The activity* was practically the same in the capillary and in the neuronal glia The variations between observations in each group after correction for autoxidation were no greater than in other parameters studied

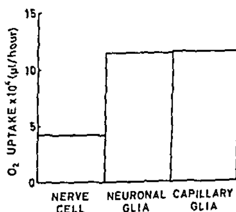


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TABLE III

Effect of hypoxia (8 % O for 15 hours) on cytochrome oxidase of nerve cell, neuronal and capillary glia of Deiters nucleus. Enzyme activity expressed as 10^{-4} µl O_2/hour. Mean values ± S.E.M.

	NERVE CELL	n	NEURONAL GLIA	n	CAPILLARY GLIA	n
CYTOCHROME OXIDASE	142 08ˣ	14	112 25	8	57 07	7

ˣ From Hamberger & Hydén 1963

The animals were placed in a glass cage and exposed to a mixture of 8% O_2 and 92% N_2 for about 15 hours. The flow rate of the gas was 3 litres/minute, the inlet and outlet were in opposite corners of the cage. The CO_2 content of the used gas was found not to exceed 0.35% as analyzed by the micro method of Scholander (1947). No pathological symptoms were observed during or after the experiment, nor did microscopic examination of samples of the brain taken up to one month later disclose any pathological changes.

The results of determinations of the cytochrome oxidase activity are given in Table III. In the nerve cell the activity was increased to almost 300% of that in the controls, whereas after vestibular stimulation the corresponding increase was 60%.

The glial reactions were the reverse of those in the previous experiment. Thus the capillary glia, formerly relatively unaffected, showed a decrease in activity after hypoxia that was nearly as great as that in the neuronal glia after vestibular stimulation. It is seen in Table III that the O_2 uptake fell to 5 · 10^{-4} µl/hour as compared to 11.6 in the control samples. The cytochrome oxidase activity in the neuronal glia was, on the contrary, practically the same as in the controls.

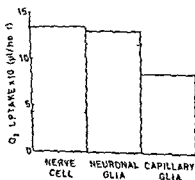


Fig. 1. Cytochrome oxidase after hypoxia.

symptoms were observed during or after stimulation One hour after the last stimulation the animal was killed and material taken for analysis

Only the cytochrome oxidase activity was assayed It is seen in Table II that vestibular stimulation produced an increase in this activity in the nerve cell to $6 \cdot 7 \times 10^{-4}$ μl O_2 per hour, as compared to 4 2 in the control samples A similar, significant increase has been recorded in succinate oxidation (Hydén & Pigon 1960)

The reaction was however most pronounced in the neuronal glia in which the activity fell from 11 5 to $3 \cdot 0 \times 10^{-4}$ μl O per hour

The activity in the capillary glia — which in the controls was the same as that in the neuronal — showed a slight decrease in the mean value i e from 11 6 to $9 \cdot 8 \times 10^{-4}$ μl O per hour Thus the response did not follow that of the neuronal glia but remained relatively unaffected by this type of stimulation This indicates that the changes which occur are strictly localized to the nerve cell with its surrounding glia

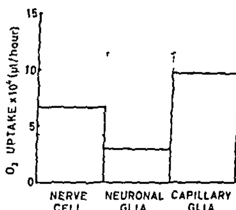


Fig 16 Cytochrome oxidase after vestibular stimulation Here and in Figs 17—20 the broken lines denote the corresponding level in the controls

Hypoxia

Nerve cells of rabbits exposed during a relatively long period to a moderate reduction in the oxygen content of the inhaled air show an increase in cytochrome oxidase activity of several hundred per cent (Hamberger & Hydén 1963) Such an adaptive change to a new environment has been demonstrated in other organs as well (Delachaux & Tissières 1946) Studies of cerebral metabolism by macrochemical methods have not given reason to expect such pronounced adjustment (Bernelli Zazzera Bassi & Cassi 1959) The degree to which the glial cells are capable of adapting their enzyme activity is therefore a matter of great interest

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Effect of hypoxia (8% O_2 for 15 hours) on cytochrome oxidase of nerve cells, neuronal and capillary glia of Deiters' nucleus. Enzyme activity expressed as 10^{-4} µl O_2/hour. Mean values ± S.E.M.

	NERVE CELL	n	NEURONAL GLIA	n	CAPILLARY GLIA	n
CYTOCHROME OXIDASE	14.2 ± 0.8[x]	14	11.2 ± 2.5	8	5.7 ± 0.7	7

x From Hamberger & Hydén 1963

The animals were placed in a glass cage and exposed to a mixture of 8% O_2 and 92% N_2 for about 15 hours. The flow rate of the gas was 3 litres/minute; the inlet and outlet were in opposite corners of the cage. The CO_2 content of the used gas was found not to exceed 0.3% as analyzed by the micro method of Scholander (1947). No pathological symptoms were observed during or after the experiment, nor did microscopic examination of samples of the brain taken up to one month later disclose any pathological changes.

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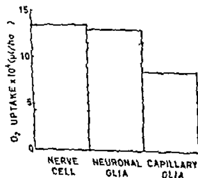


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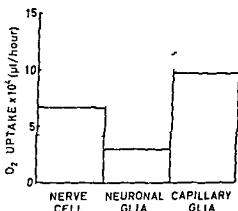


Fig 16 Cytochrome oxidase after vestibular stimulation Here and in Figs 1 — 20 the broken lines denote the corresponding level in the controls

Hypoxia

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	NERVE CELL	n	NEURONAL GLIA	n	CAPILLARY GLIA	n
CYTOCHROME OXIDASE	4 08[x]	7	11.2 25	8	5.7 07	7

x From Hamberger & Hydén 1963

The animals were placed in a glass cage and exposed to a mixture of 8% O_2 and 92% N_2 for about 15 hours. The flow rate of the gas was 3 litres/minute, the inlet and outlet were in opposite corners of the cage. The CO_2 content of the used gas was found not to exceed 0.35% as analyzed by the micro method of Scholander (1947). No pathological symptoms were observed during or after the experiment, nor did microscopic examination of samples of the brain taken up to one month later disclose any pathological changes.

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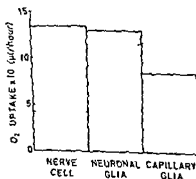


Fig. 17. Cytochrome oxidase after hypoxia.

symptoms were observed during or after stimulation One hour after the last stimulation the animal was killed and material taken for analysis

Only the cytochrome oxidase activity was assayed It is seen in Table II that vestibular stimulation produced an increase in this activity in the nerve cell to 6.7×10^{-4} μl O_2 per hour, as compared to 4.2 in the control samples A similar significant increase has been recorded in succinate oxidation (Hydén & Pigon 1960)

The reaction was however most pronounced in the neuronal glia, in which the activity fell from 11.5 to 3.0×10^{-4} μl O_2 per hour

The activity in the capillary glia — which in the controls was the same as that in the neuronal — showed a slight decrease in the mean value i e, from 11.6 to 9.8×10^{-4} μl O_2 per hour Thus the response did not follow that of the neuronal glia but remained relatively unaffected by this type of stimulation This indicates that the changes which occur are strictly localized to the nerve cell with its surrounding glia

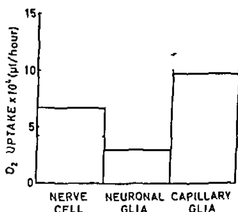


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In order to study the effect of TRIAP on α ketoglutarate oxidation in the capillary endothelium I made a few experiments with small bundles of the finest branches after freeing them carefully from all glial tissue (It must be pointed out that estimation of the volume was less exact than in the rest of the material) No noteworthy difference was present between the O_2 uptake in samples from treated animals and from controls

Some experiments were also performed to ascertain the effect of TRIAP *in vitro* i e added to the incubation medium instead of being injected into the animal Cytochrome oxidase activity was found to be markedly inhibited at a concentration of 0 001 and 0 01 mg/ml and slightly stimulated at 0 1 mg/ml α Ketoglutarate oxidation was somewhat decreased at the two higher concentrations a little more at the highest No difference was noticeable between the *in vitro* effect on nerve cells and on glial cells It did not seem possible to reproduce the dramatic changes observed after intravenous administration in any event with the concentrations used

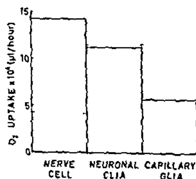


Fig 18

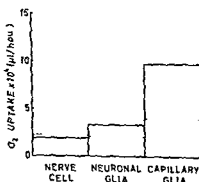


Fig 19

Fig 18 Cytochrome oxidase after TRIAP

Fig 19 α Ketoglutarate oxidation after TRIAP

Dinitrophenol

It can be inferred from the preceding section that the quantitative and qualitative changes in the RNA content of nerve cells and glial cells induced by TRIAP were in fact accompanied by a complicated pattern of profound changes in their enzyme activity Moreover this substance has been shown to cause uncoupling of oxidative phosphorylation in the liver mitochondria of the rabbit (Eberts 1960) I therefore considered it desirable to compare

Tricyano Amino Propene

Hydén (1947) showed that administration of malononitrile *to rabbits raised* the RNA and protein content of nerve cells. The active substance in the preparation used has subsequently been identified as 1 1 3 tricyano 2 amino 1 propene (TRIAP)[1] a dimer of malononitrile (Eberts 1960). *As mentioned in* Chapter I, certain chemical agents — among them TRIAP — have proved to exert a strong action on RNA and protein synthesis in nerve cells and glial cells of experimental animals.

Gomirato Ferro Milone & Martinelli (1961) tested TRIAP in human subjects. No toxic symptoms were observed *even after doses greatly in excess of* those used in animal experiments.

In view of these findings I considered it profitable to study the effect of TRIAP to obtain data on the response of the nervous tissue to rapid changes in RNA synthesis. TRIAP was injected intravenously, the dose being 20 mg/kg of body weight of an aqueous solution containing 40 mg/ml. A slight rise in temperature was recorded, but no other toxic symptoms. The animals were killed one hour after the injection.

The results are listed in Table IV. It is seen that the cytochrome oxidase activity in the nerve cell $13\ 4 \times 10^{-4}$ μl O per hour rose to almost the same level as after hypoxia. Although the RNA content of the neuronal glia decreased by 45% — *with an associated change in the ba e composition* (E[illegible] and Hydén 1961) — its cytochrome oxidase activity remained practically unaltered. That of the capillary glia was reduced to $8\ 4 \times 10^{-4}$ μl hour; the decrease was not, however, as great as after hypoxia.

As far as α ketoglutarate oxidation is concerned, the changes induced were partly *in the opposite direction. Thus the activity in both nerve cell* and neuronal glia showed an inappreciable change. The only component of the model system to be affected was the capillary glia, in which the activity was *increased by more than 100%*.

TABLE IV

Effect of tricyano amino propene on enzyme activity of nerve cell, neuronal and capillary glia of Deiters nucleus. 20 mg/kg injected i.v. 1 hour before death. Enzyme activity expressed as 10^{-4} μl O_2/hour. Mean values ± S.E.M.

	NERVE CELL	n	NEURONAL GLIA	n	CAPILLARY GLIA	n
CYTOCHROME OXIDASE	134 12	11	129±16	9	84 08	11
α-KETOGLUTARATE OXIDATION	19±04	11	33 06	12	97 19	13

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1 TRIAP was kindly supplied by *The Upjohn Company*, Kalamazoo, Mich., U.S.A.

In order to study the effect of TRIAP on α ketoglutarate oxidation in the capillary endothelium I made a few experiments with small bundles of the finest branches after freeing them carefully from all glial tissue (It must be pointed out that estimation of the volume was less exact than in the rest of the material.) No noteworthy difference was present between the O_2 uptake in samples from treated animals and from controls

Some experiments were also performed to ascertain the effect of TRIAP *in vitro* i e added to the incubation medium instead of being injected into the animal Cytochrome oxidase activity was found to be markedly inhibited at a concentration of 0 001 and 0 01 mg/ml and slightly stimulated at 0 1 mg/ml α ketoglutarate oxidation was somewhat decreased at the two higher concentrations a little more at the highest No difference was noticeable between the *in vitro* effect on nerve cells and on glial cells It did not seem possible to reproduce the dramatic changes observed after intravenous administration in any event with the concentrations used

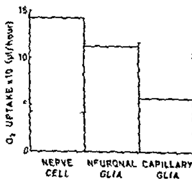


Fig 18

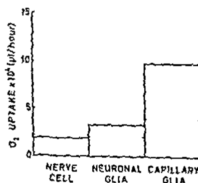


Fig 19

Fig 18 Cytochrome oxidase after TRIAP

Fig 19 α Ketoglutarate oxidation after TRIAP

Dinitrophenol

It can be inferred from the preceding section that the quantitative and qualitative changes in the RNA content of nerve cells and glial cells induced by TRIAP were in fact accompanied by a complicated pattern of profound changes in their enzyme activity Moreover this substance has been shown to cause uncoupling of oxidative phosphorylation in the liver *mitochondria* of the rabbit (Eberts 1960) I therefore considered it desirable to compare

Tricyano Amino Propene

Hydén (1947) showed that administration of malononitrile to rabbits raised the RNA and protein content of nerve cells. The active substance in the preparation used has subsequently been identified as 1,1,3-tricyano-2-amino-1-propene (TRIAP)[1], a dimer of malononitrile (Eberts 1960). As mentioned in Chapter I, certain chemical agents — among them TRIAP — have proved to exert a strong action on RNA and protein synthesis in nerve cells and glial cells of experimental animals.

Gomirato, Ferro-Milone & Martinelli (1961) tested TRIAP in human subjects. No toxic symptoms were observed even after doses greatly in excess of those used in animal experiments.

In view of these findings I considered it profitable to study the effect of TRIAP to obtain data on the response of the nervous tissue to rapid change in RNA synthesis. TRIAP was injected intravenously, the dose being 20 mg/kg of body weight of an aqueous solution containing 40 mg/ml. A slight rise in temperature was recorded but no other toxic symptoms. The animals were killed one hour after the injection.

The results are listed in Table IV. It is seen that the cytochrome oxidase activity in the nerve cell, 13.4×10^{-4} μl O_2 per hour, rose to almost the same level as after hypoxia. Although the RNA content of the neuronal glia decreased by 45% — with an associated change in the base composition (Egyházi and Hydén 1961) — its cytochrome oxidase activity remained practically unaltered. That of the capillary glia was reduced to 8.4×10^{-4} μl/hour; the decrease was not, however, as great as after hypoxia.

As far as α-ketoglutarate oxidation is concerned, the changes induced were partly in the opposite direction. Thus, the activity in both nerve cell and neuronal glia showed an inappreciable change. The only component of the model system to be affected was the capillary glia, in which the activity was *increased by more than 100%*.

TABLE IV

Effect of tricyano-amino-propene on enzyme activity of nerve cell, neuronal and capillary glia of Deiters' nucleus. 20 mg/kg injected i.v. 1 hour before death. Enzyme activity expressed as 10^{-4} μl O_2/hour. Mean value ± S.E.M.

	NERVE CELL	n	NEURONAL GLIA	n	CAPILLARY GLIA	n
CYTOCHROME OXIDASE	13.4 ± 1.2	11	12.9 ± 1.6	9	8.4 ± 0.8	11
α-KETOGLUTARATE OXIDATION	1.9 ± 0.4	11	3.3 ± 0.6	12	9.7 ± 1.9	13

[1] TRIAP was kindly supplied by The Upjohn Company, Kalamazoo, Mich., U.S.A.

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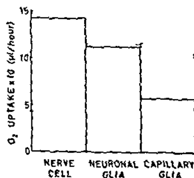


Fig 18

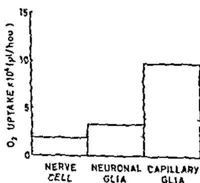


Fig 19

Fig 18 Cytochrome oxidase after TRIAP

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It can be inferred from the preceding section that the quantitative and qualitative changes in the RNA *content* of nerve cells and glial cells induced by TRIAP were *in fact* accompanied by a complicated pattern of profound changes in their enzyme activity Moreover this substance has been shown to cause uncoupling of oxidative phosphorylation in the liver mitochondria of the rabbit (Eberts 1960) I therefore considered it desirable to compare

TABLE V

Effect of 2 4 dinitrophenol on α ketoglutarate oxidation by nerve cells neuronal and capillary glia of Deiters nucleus Rabbit killed one hour after injection At 2 5 and 12 5 mg/kg single determination at 5 mg/kg mean of 2 determinations Enzyme activity expressed as 10^{-4} µl O_2/hour

2 4 DINITROPHENOL mg/kg	NERVE CELL	NEURONAL GLIA	CAPILLARY GLIA
2.5	0.6	2.2	3.8
5.0	11.2	14.4	12.3
12.5	4.0	2.3	3.6

the effect of TRIAP with that of another uncoupling agent in an attempt to determine the nature of any typical reactions in the relevant cell types For this purpose a few experiments were performed with 2 4 dinitrophenol (DNP) a substance with a well established *in vitro* effect

DNP was administered by intravenous injection of an aqueous solution of the sodium salt, containing 4 mg/ml The injection was given in the course of about 1 minute in doses of 2 5 5 0 and 12 5 mg/kg of body weight In addition to hyperpyrexia some motor restlessness was noticed with the highest dose The animals were killed one hour after the injection

The results are shown in Table V After the lowest and highest dose (1 animal each) *α ketoglutarate oxidation in the neuronal and capillary glia was unaffected* Although a decrease and an increase respectively were recorded in the nerve cell they seemed more likely to be a result of the variation between individual observations than an indication of a *definite change in activity*

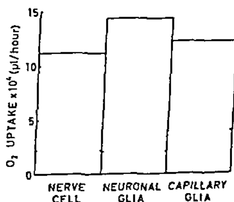


Fig 20 α ketoglutarate oxidation after DNP (mg/kg)

After a dose of 5 mg/kg (2 animals) on the contrary the increase in oxygen uptake was striking It amounted to at least 300% and in every case exceeded the highest value in the controls The specific feature was that an increase in activity took place in all three components of the system indicating that the mechanism of action of DNP differs from that of TRIAP as well as that of the more physiological stimuli The effect of DNP has a considerably less complicated distribution even if the factors underlying it are uncertain

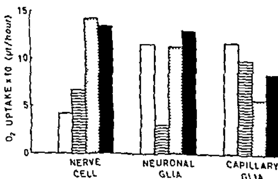


Fig. 21 Summary of cytochrome oxidase assays

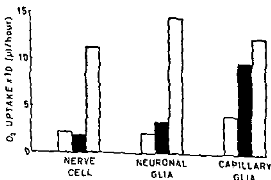


Fig. Summary of α-ketoglutarate oxidation assays

CONTROLS
VESTIBULAR STIMULATION
HYPOXIA
TRICYANOAMINOPROPENE
DINITROPHENOL

TABLE V

Effect of 2 4 dinitrophenol on α ketoglutarate oxidation by nerve cells neuronal and capillary glia of Deiters nucleus Rabbit killed one hour after injection At 2 and 12 mg/kg single determination at 5 mg/kg mean of 2 determinations *Enzyme activity expressed as* 10^{-4} μl O_2/hour

2 4-DINITROPHENOL mg/kg	NERVE CELL	NEURONAL GLIA	CAPILLARY GLIA
2 5	0 6	2 2	3 8
5 0	11 2	14 4	12 3
12 5	4 0	2 3	3 6

the effect of TRIAP with that of another uncoupling agent, in an attempt to *determine the nature of any typical reactions in the relevant cell types* For this purpose, a few experiments were performed with 2,4 dinitrophenol (DNP) a substance with a well established *in vitro* effect

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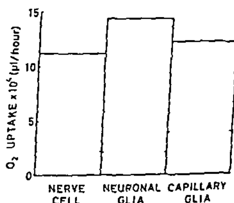


Fig 20 *α Ketoglutarate oxidation after DNP (5 mg/kg)*

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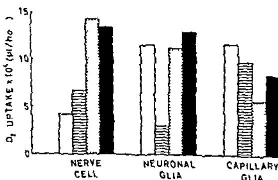


Fig 1 Summary of cytochrome oxidase assay

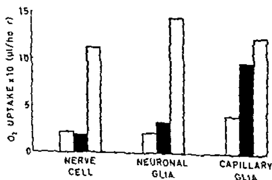


Fig Summary of α ketoglutarate oxidation assays.

- CONTROLS
- VESTIBULAR STIMULATION
- HYPOXIA
- TRICYANOAMINOPROPENE
- DINITROPHENOL

TABLE V

Effect of 2 4 dinitrophenol on α ketoglutarate oxidation by nerve cells neuronal and capillary glia of *Deiters nucleus* Rabbit killed one hour after injection At 2 and 12 5 mg/kg single determination at 5 mg/kg mean of 2 determinations Enzyme activity expressed as 10^{-4} µl O_2/hour

2 4-DINITROPHENOL mg/kg	NERVE CELL	NEURONAL GLIA	CAPILLARY GLIA
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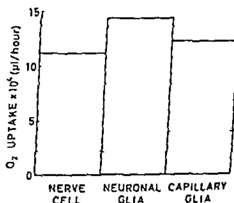


Fig 20 α Ketoglutarate oxidation after DNP (5 mg/kg)

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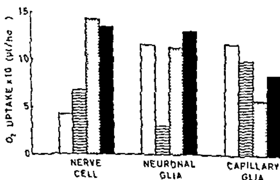


Fig. 1 Summary of cytochrome oxidase assays

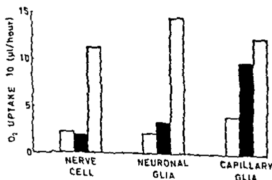


Fig Summary of α ketoglutarate oxidation assays

- CONTROLS
- VESTIBULAR STIMULATION
- HYPOXIA
- TRICYANOAMINOPROPENE
- DINITROPHENOL

TABLE V

Effect of 2,4 dinitrophenol on α ketoglutarate oxidation by nerve cells, neuronal and capillary glia of Deiters nucleus. Rabbit killed one hour after injection. At 2.5 and 12.5 mg/kg single determination, at 5 mg/kg mean of 2 determinations. Enzyme activity expressed as 10^{-4} µl O_2/hour

2,4 DINITROPHENOL mg/kg	NERVE CELL	NEURONAL GLIA	CAPILLARY GLIA
2.5	0.6	2.2	3.8
5.0	11.2	14.4	12.3
12.5	4.0	2.3	3.6

the effect of TRIAP with that of another uncoupling agent in an attempt to determine the nature of any typical reactions in the relevant cell types. For this purpose a few experiments were performed with 2,4 dinitrophenol (DNP), a substance with a well established *in vitro* effect.

DNP was administered by intravenous injection of an aqueous solution of the sodium salt containing 4 mg/ml. The injection was given in the course of about 1 minute in doses of 2.5, 5.0 and 12.5 mg/kg of body weight. In addition to hyperpyrexia some motor restlessness was noticed with the highest dose. The animals were killed one hour after the injection.

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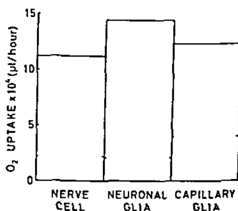


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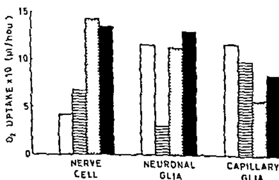


Fig 1 Summary of cytochrome oxidase assays

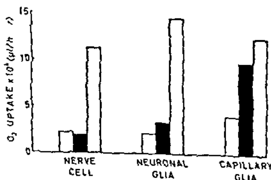


Fig 2 Summary of α ketoglutarate oxidation assays

CONTROLS
VESTIBULAR STIMULATION
HYPOXIA
TRICYANOAMINOPROPENE
DINITROPHENOL

TABLE V

Effect of 2 4 dinitrophenol on α ketoglutarate oxidation by nerve cells neuronal and capillary glia of Deiters nucleus Rabbit killed one hour after injection At 2 5 and 12 5 mg/kg single determination at 5 mg/kg mean of 2 determinations Enzyme activity expressed as 10^{-4} µl O_2/hour

2 4-DINITROPHENOL mg/kg	NERVE CELL	NEURONAL GLIA	CAPILLARY GLIA
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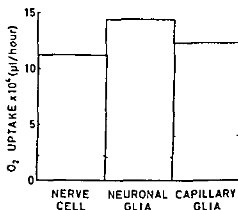


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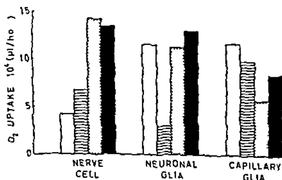


Fig 1 Summary of cytochrome oxidase assays

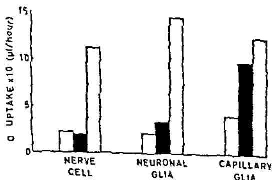


Fig 2 Summary of α ketoglutarate oxidation assays

CONTROLS
VESTIBULAR STIMULATION
HYPOXIA
TRICYANOAMINOPROPENE
DINITROPHENOL

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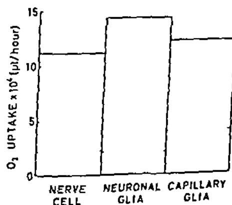


Fig 20 α ketoglutarate oxidation after DNP (5 mg/kg)

CHAPTEP IV

GENERAL DISCUSSION

The present investigation comprised the following

1 Separation of glial populations on the micro scale in order to produce a biological model system of the grey matter in the brain

2 Attempts to characterize the different components of the system biochemically

3 Attempts to produce specific chemical effects on the components by means of exogeneous stimuli

Separation of Glial Populations

I used a micro dissection technique to separate the neuroglial populations in an attempt to illustrate in a concrete way the classical views on the relation of the glial cells to other elements of the central nervous system

The nerve cell was used as the standard volume and was analyzed as the first component of the system This was because the biochemical activity and reactions of the glial cells must be seen against the background of events taking place in the nerve cell

The second component the neuronal glia has been analyzed previously on the cellular level and compared with the nerve cell

The third component the capillary glia — on which mo t attention was focused in this study — has not been analyzed before by quantitative methods The question of particular interest was to what extent incorporation of this component in the system would provide a more detailed conception of the behaviour of the central nervous system on the cellular level

Studies of histological sections have led to certain conclusions regarding the intimacy of the relation between glial cells and neuron and blood vessel respectively In micro dissection of fresh tissue in the present study the mechanical contact observed was used as the criterion of the glial cells relation to their surroundings

In the lateral vestibular nucleus of the rabbit the RNA content of the glial cells is about 10% of that of the nerve cells on a dry weight basis (Hydén & Pigon 1960) Since the neuroglia is responsible for a great proportion of the volume of the grey matter a large contribution must be made by its cells to the total amount of RNA I therefore considered it of interest to compare the two glial systems from the point of view of their total RNA content

The determination of RNA in $\mu\mu$g per volume corresponding to that of a nerve cell was performed according to Edstrom (1958) The RNA was extracted from the cells with a buffered ribonuclease solution The RNA content of the extracts was determined in micro drops by a photographic photometric method with ultraviolet light at 2570 Å

The results are given in Table VI It is seen that the total amount of RNA in the capillary glia was about 10% less than in the neuronal The difference is slight and may reflect differences between the distribution of cell types in the two populations

TABLE VI

RNA content ($\mu\mu$g/sample) of neuronal and capillary glia of Deiters nucleus Mean values ± S E M

NEURONAL GLIA	n	CAPILLARY GLIA	n
150 41	4	138±4 5	4

α ketoglutarate and glutamate amounted to 2.2×10^{-4} μl O_2 per hour with approximately twice the value for cytochrome oxidase. The dry weight of nerve cells and glial samples as used in the present investigation is about 20 000 μμg (Hyden & Pigon 1960). This implies a Q_O (μl/mg dry weight/hour) of around 10.

In the control samples the respiration of the neuronal glia was the same as that of the nerve cell when α ketoglutarate was used as substrate, twice as large in succinate and half as large in glutamate. The cytochrome oxidase activity was about three times that in the nerve cell.

The oxygen uptake in the capillary glia was twice as high as in the nerve cell on incubation with α ketoglutarate, three times as high with succinate and somewhat lower with glutamate. The cytochrome oxidase activity — like that in the neuronal glia — was three times as great as in the nerve cell.

Consequently the Q_{O_2} of the neuronal glia was 5—20 and that of the capillary glia 8—30 depending on the substrate oxidized. This denotes an average quotient of approximately 14 for the whole system, a figure in fairly good agreement with that reported in macrochemical assays in slices or homogenates (Krebs 1950).

These data on the relation nerve cell/glia are to some extent at variance with the calculations made by Elliott & Heller (1957) and Korey & Orchen (1959) on the basis of macrochemical analyses since the present figures indicate greater metabolic activity in the glia. Korey & Orchen calculated the respiration per glial cell (corpus callosum) to be around 1.2×10^{-5} μl O_2/hour, a figure practically identical with the lowest recorded in the present study, namely in glutamate oxidation by neuronal glia. The highest value recorded, i.e. that for succinate oxidation by capillary glia, is roughly seven times higher than their value. Korey, Orchen & Brotz (1958) also found that succinate was of importance for high glial respiration and that glutamate enhanced the activity in nerve cells.

Qualitative histochemical studies generally show the nerve cell bodies to have by far the highest content of respiratory enzymes (Thomas & Pearse 1961, Friede 1962). Quantitative micro analyses suggest, on the contrary, that in certain cases the enzyme activity of the glial cells is greater than that of the nerve cells. According to Robins (1960) the nerve cell bodies usually have lower activity than other parts of the grey matter. Similar results were reported by Buell *et al.* (1958) in analyses of the cerebellum and Ammon's horn. Lowry's studies (1957 a, b) on isolated ganglion cells and glial capsules showed higher activity in the nerve cells of enzymes implicated in glutamic acid metabolism, an observation in agreement with my finding of glutamate being the only substrate that was oxidized more rapidly by the nerve cells than by the

Studies of fixation artefacts in nervous tissue (Bairati 1958) give evidence of the tenacity of the glial cells contacts especially as far as vessels are concerned From a more functional point of view additional proof of the close contact between glia and vessel has been provided by the observations of R Geiger (1962) in cultures of nervous tissue when a little phenol red was added to the medium The capillary cement concentrated the dye and the sucker processes of the astrocytes in contact with vessels concentrated it further and took it up in the cell body The astrocytes that were not in contact with vessels showed no trace of the dye In addition to the morphological connexions that have been observed as mentioned in Chapter I the exceedingly adherent capsule of the spinal ganglion cells is an example of the mechanical relation between glial cells and the neuron

As shown in Chapter II the capillary glia contains more astrocytes than the neuronal This implies a theoretical possibility of establishing the relative metabolism in the astrocytes and oligodendrocytes a problem that has been extensively studied over a period of years This problem must however remain more or less unanswered in the present investigation since it is not known whether the metabolism of the oligodendrocytes is the same in the different glial populations

A factor of some importance in this type of investigation is the purity of the glial samples With the technique I used this source of error has proved to be small

Another matter of interest is the degree of vitality of the samples Dissected out nerve cells and glial cells from Deiters nucleus have been kept for many months in a fluid nutrient medium without any apparent morphological signs of cell degeneration (Hansson & Sourander personal communication) The degree of persisting function in the cells is hard to define The fact that the cells can be seen under the phase contrast microscope to have an unchanged structure after 24 hours in the incubation medium suggests that their structural organization has been retained during the experiment and that in this respect the cell is not merely a bag of mitochondria Actually the question is not of any major practical importance for the analyses in question here as even a bag of mitochondria' can be presumed to reflect the enzyme content of the cell at death of the animal

Characterization of the System s Components

Certain points must be mentioned regarding the nerve cell which served as the standard volume in dissection of the glia Its metabolism varied less than in the other control material Thus the oxidation of succinate as well as of

culation see p 22) Using the results of succinate oxidation (Table 1) we get the following

7 oligodendrocytes+1 astrocyte=4 9
6 oligodendrocytes+2 astrocytes=6 9

This implies that an astrocyte would have about 15 times higher activity than an oligodendrocyte The corresponding figure for α ketoglutarate oxidation would be around 20

Although this would be an interesting result it must for the time being remain a speculation to some extent because of uncertainty in identifying the different types of neuroglial cell (cf p 22) Moreover it is in contradiction to the majority of observations made in this field with other techniques

Many workers who have studied the blood brain barrier have stressed the important role played by the pericapillary glia in maintaining the permeability (e g De Robertis & Gerschenfeld 1961) Such a function could account for its higher oxygen uptake

To sum up the following statement can be made regarding the present results in the controls With few exceptions the enzyme activity of the glial cells was higher than that of the nerve cells With one exception i e cyto chrome oxidase the capillary glia showed greater activity than the neuronal The metabolic activity gradient from vessel to nerve cell can possibly be envisaged as parallel to an oxygen gradient in the same direction

Exogenous Stimulation

It must be emphasized that in the present connexion the term *stimulation* denotes incitement to a change in neuronal function Hydén (1963) concluded that An increased amount of RNA per nerve cell measured during a period of time can be taken as an indicator of increased neuronal function

Vestibular stimulation

The first type of stimulation used in the present study was vestibular stimulation by rotation of the animal In the nerve cells of Deiters nucleus this produces an increase in the cytochrome oxidase RNA and protein content and a decrease in anaerobic glycolysis with changes in the reverse direction in the neuronal glia (Hydén & Pigon 1960 Hamberger & Hyden 1963) A kinetic study of succinoxidase activity and its temperature dependence under the same experimental conditions showed a difference between the neuron

glial cells Lowry stated that the activity of lactic dehydrogenase and isocitric dehydrogenase is the same in nerve cells and glia whereas that of glycolytic enzymes is greater in the glial cells

The aforementioned macrochemical analyses as well as the histochemical staining reactions complement the present results even if certain marked discrepancies are difficult to explain at this stage They can presumably be ascribed partly to variations in the relative activity in different parts of the CNS

The relation between the adenosine triphosphatase activity of the neuronal and capillary glia has recently been investigated by Cummins & Hydén (1962) in samples dissected out exactly as in the present study The activity was 50% higher in the neuronal glia indicating an inverse relation between oxygen uptake and ATPase activity in this case

The possibility that the difference between the ratio of astrocytes/oligodendrocytes in capillary and neuronal glia may actually be responsible for the present findings with respect to enzyme activity is to some extent borne out in the literature Thus Nasu & Viale (1961) found considerably greater lactic dehydrogenase, *DPNH* and *TPNH* diaphorase activity in astrocytomas than in oligodendrogliomas in histochemical studies with tetrazolium salts Lehrer (1961) using Lowry s microchemical assay method demonstrated high activity of *e g* isocitric dehydrogenase particularly in well differentiated astrocytomas Allen (1957) reported on the other hand that the oligodendroglioma contained eight times as much cytochrome oxidase as the astrocytoma Respiratory determinations have given similar results (Elliott & Heller 1957) *It must however be recalled that even if tumours do represent pure glial* samples it is difficult to state to what extent the relative activity in different types reflects the activity in organized tissue

Histochemical determinations in sections of normal material suggest that under normal conditions the astrocyte is the least enzyme rich of all cells in the central nervous system (Friede 1962) Rubinstein *Klatzo & Miquel* (1962) found low activity in the glial cells of the white matter without any *apparent difference between the two types of glia*

The differences found in the present study between the enzyme activity of the neuronal and capillary glia are striking and may be a function either of their location or of their composition with respect to cell types It is difficult to enter into any discussion of the former alternative If on the other hand the results are a function of composition a rough estimate can be made of the difference between the enzyme activity of astrocytes and oligodendrocytes

Let us assume that on the average the neuronal glia contains 1 astrocyte per 8 glial cells and the capillary glia 2 astrocytes per 8 glial cells (for cal

culation see p 22) Using the results of succinate oxidation (Table I) we get the following

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and the neuronal glia The neuron increased its activity as a function of stimulation The glia did not change in this respect nor did the calculated activation energy of the reaction reveal any temperature dependence within the range 37° to 27° C (Hyden & Lange 1962)

Enhanced activity in a tissue *e g* nervous tissue is accompanied by an increase in its utilization of oxygen and glucose (Larrabee 1961) In the system in question here, it is evidently the nerve cell that chiefly shows a tendency to a raised oxygen uptake and the neuronal glia to an increase in the capacity to utilize glucose This indicates that nerve cell and neuronal glia form a unit whose reactions are complementary No decrease in cytochrome oxidase activity was however observed in the capillary glia A possible cause is its more intimate contact with the oxygen and nutrient transporting blood In this case the capillary glia is to a certain extent unattached to the unit formed by nerve cell plus neuronal glia

If it is presumed that the decrease in activity after stimulation involves only the oligodendrocytes the larger number of astrocytes in the capillary glia would explain why the decrease is confined to the perineuronal glial tissue Friede (1962) has in fact demonstrated that the astrocytes have the ability to react in the form of increased enzyme activity to a greater degree than the oligodendrocytes and even than the nerve cells Rubinstein Klatzo & Miquel (1962) also reported enormous astrocyte activity with an increase in various respiratory enzymes after experimental brain damage whereas no change could be observed in the oligodendrocytes On the other hand Koenig & Barron (1962) found that an experimental demyelinating lesion of the spinal cord produced enlargement and proliferation of the oligodendrocytes and that the reactive glial cells had a raised RNA content and enhanced reactions to oxidative enzymes

For the time being it is only possible to speculate on the causes of the changes in enzyme activity in the system

Hypoxia

To permit a comparison with the reactions of the system under different environmental conditions a series of rabbits were exposed to air with an oxygen content of 8% I also hoped that this might help to explain the changes in enzyme activity produced by vestibular stimulation

With this reduction of the oxygen level in the inhaled air the cerebral metabolism proceeds largely unaltered the lactic acid production is slightly raised but the ATP and phosphocreatine levels are unaffected (Gurdjian

Webster & Stone 1949) In the present experiments the nerve cell reacted in the same way as to vestibular stimulation namely by an increase in cytochrome oxidase activity although it was a much larger one The glial reactions were on the contrary the reverse of those in the previous experiment i e the activity in the neuronal glia was unchanged and that in the capillary glia increased This implies that a similar response is elicited from the nerve cell irrespective of whether stimulation involves increased activity or lowered O_2 tension The earlier reciprocity indicating interaction of nerve cell and neuronal glia was no longer present

Little is known about the effect of prolonged hypoxia on cerebral metabolic processes Since most experiments have been made under purely anoxic conditions no pronounced adaptive changes on the cellular level have had time to occur Severe anoxia of various kinds causes a decrease in oxygen uptake (Rosenthal Shenkin & Drabkin 1945) As the animals survival is prolonged by barbiturate anaesthesia and/or exposure to anoxia prior to anoxic experiments (Cahn *et al* 1961) certain metabolic processes in the brain can be modified to lessen the effect of reduced O_2 tension

Bernelli Zazzera Bassi & Cassi (1959) exposed rats to a hypoxic atmosphere (3% O_2 and 97% N_2) for two hours Analysis of brain slices disclosed no changes in respiration respiratory quotient phosphorylation processes or amino acid incorporation In my experiments the sum of activity changes in the system was small due to concurrent positive and negative alterations This implies that the results are not necessarily in opposition to those of *Bernelli Zazzera et al* In guinea pigs adapted to low oxygen tension during a long period Delachaux & Tissieres (1946) found a 250–300% increase in cytochrome *c* in the skeletal muscle which is much more homogeneous than cerebral tissue

The structural elements of the brain are sensitive to hypoxia in the following order 1) neuron 2) oligodendrocyte 3) astrocyte 4) microglia 5) connective tissue (Lewis & Swank 1953 Scholz 1958) A reaction in the form of enhanced enzyme activity seems to occur only when the sensitivity is great enough for hypoxia to act as a stimulus The difference between the reaction of the neuronal and the capillary glia might be explained by the higher astrocyte content of the latter

The low oxygen uptake in cells surrounding the capillaries may imply some kind of parallel phenomenon to the reaction of the neuronal glia after vestibular stimulation i e a depressive effect to save the already limited oxygen supply for the needs of the nerve cell For glial tissue has a considerably greater glycolytic capacity (Victor & Wolf 1937 Elliott & Heller 1957 Hamberger & Hydén 1963)

Tricyano amino propene

The enzymic changes occurring after vestibular stimulation and hypoxia were accompanied by changes in the RNA and protein content of the cells. Several types of stimulation involving increased functional demands on the nerve cell produce marked alterations in its RNA content (Hydén 1962). I therefore studied the changes in enzyme activity that have been found to result from chemically induced (TRIAP) rapid pronounced changes in RNA and protein content (Egyhazi & Hydén 1961).

With respect to cytochrome oxidase the results were similar to those after hypoxia. α-Ketoglutarate oxidation was influenced in the opposite direction, *i.e.*, the activity was practically unchanged in the nerve cell and neuronal glia, whereas it was raised in the capillary glia.

After vestibular stimulation the changes in cytochrome oxidase activity are associated with parallel changes in succinoxidase and RNA content in both glial cells and nerve cells (Hydén & Pigon 1960). As far as TRIAP is concerned, it appears that α-ketoglutarate oxidation does not necessarily run parallel to the changes in cytochrome oxidase.

Measurements of the endogenous respiration extrapolated to the time of the animal's death show that it is totally inhibited in both nerve cells and neuronal glia by TRIAP (Hydén & Lange in press). It seems that oxidation of the substrate, represented here by α-ketoglutarate, is inhibited during the TRIAP induced synthesis of RNA. A quantitative increase in RNA content as in *the nerve cell is evidently accompanied by raised cytochrome oxidase* activity, whereas a quantitative decrease as in the neuronal glia does not induce any change in the activity of this enzyme.

The capillary glia *which exhibited pronounced changes in activity* of both enzymes assayed differed completely in behaviour from the neuronal glia which was almost unaffected.

Dinitrophenol

In vitro TRIAP causes uncoupling of oxidative phosphorylation in liver mitochondria (Eberts 1960). To ascertain whether this mechanism of action might have been responsible for the increased respiration in the capillary glia the effect on α-ketoglutarate oxidation of dinitrophenol injected intravenously before death of the animal was investigated (Table V). In a system with respiratory control DNP usually produces an increased oxygen uptake by uncoupling the phosphorylation processes associated with oxidation (Loomis & Lipmann 1948). This does not *however apply to brain mitochondria* (Lar

trup & Svennerholm 1963) When injected into animals the effect of DNP is expressed as a rise in basal metabolism and body temperature (Slater & Hulsmann 1959) although no changes in the activity of respiratory enzymes have been observed in tissue slices (Dianzani & Scuro 1956) In the present investigation on the contrary distinct changes in activity were seen after a suitable dose all the components of the system being influenced in the same direction

Several factors may account for this difference between the effect of TRIAP and DNP For example the lipid solubility of a substance is considered to play a role as far as penetration into the cerebral tissues is concerned (Mayer Maickel & Brodie 1959 for bibliography see Lajtha 1962) Tests of the lipid solubility expressed as the organic solvent/water partition coefficient do not however show any definite difference between TRIAP and DNP in this respect No explanation of the effect of DNP can in fact be given when such a complicated system as a whole animal is involved The only permissible conclusion is that TRIAP has an effect similar to that of dinitrophenol on the capillary glia whereas the other components of the system are not influenced in this direction

Conclusions

The response of the nerve cell to the types of stimulation tested here was in the form of a significant increase in cytochrome oxidase activity RNA and protein content which was however considerably greater after hypoxia and TRIAP than after vestibular stimulation Respiration was not on the contrary increased after TRIAP when α ketoglutarate was used as substrate The neuronal glia did not as a rule react as far as the variables investigated were concerned with the exception of a marked decrease in activity after vestibular stimulation Its RNA content seemed however to be more easily influenced

In no case did either the neuronal or capillary glia respond by an increase in cytochrome oxidase activity In the capillary glia on the other hand oxidation of the substrate could be influenced in a positive direction

In earlier studies of the effect of vestibular stimulation inverse changes in nerve cell and neuronal glia were observed with respect to both cytochrome oxidase and anaerobic glycolysis (Hydén & Lange 1960 Hamberger & Hydén 1963) It was therefore concluded that these two components form a functional metabolic unit

When the reactions to the other stimuli used in the present investigation are studied it is seen that reciprocal changes occur in nerve cell and capillary

glia instead It is difficult to decide whether these results imply that in certain cases the nerve cell forms a unit with the neuronal glia, and in others with the capillary glia One is more inclined to interpret the reactions as an indication that, in certain cases the neuronal glia can be shown to have a metabolic relation to the nerve cell The capillary glia should — in analogy with the neuronal — be evaluated in relation to the capillary endothelium which is the borderline tissue between blood and brain The relatively high enzyme activity, as well as the changes in connexion with stimulation shown to be specific to capillary glia, demonstrate that this tissue element participates most strikingly in the metabolic activities of the nervous system

SUMMARY

The present investigation consists of separation and biochemical characterization of glial populations on the micro scale The main object is to study the glia surrounding the finest cerebral vessels the *capillary glia* This component is compared with the glia surrounding nerve cells *i e* the *neuronal glia* and with *nerve cells* Cells within the lateral vestibular nucleus of Deiters in the rabbit are used for this purpose

The biochemical characterization implies analyses of glial clumps containing 7–8 cells (approximately 10^{-8} g of tissue) as well as isolated nerve cells The oxidation of cytochrome c succinate *α ketoglutarate and glutamate* is determined by measuring the oxygen uptake with the micro diver technique of Zeuthen (1953)

In the controls the nerve cell shows the lowest enzyme activity per volume unit or per unit dry weight except for glutamate oxidation The activity of the capillary glia is found to be 50–100 % higher than that of the neuronal apart from cytochrome oxidase whose activity is similar in both types of glia

After experimental stimulation of the animals — by rotation hypoxia and chemical means — the *nerve cell* reacts in every case by an increase in cytochrome oxidase activity The response of the two glial components is found to differ

The *capillary glia* is uninfluenced by vestibular (rotatory) *stimulation* whereas after hypoxia and chemical stimulation (TRIAP) the change is the reverse of that in the nerve cell *i e* a decrease in cytochrome oxidase activity Moreover an increase in α ketoglutarate oxidation is observed after chemical stimulation (TRIAP) in contrast to the practically unaltered activity *of this* enzyme in the nerve cell

The *neuronal glia* is found *to* react to vestibular stimulation by a decrease in cytochrome oxidase activity As regards the other factors studied *it seems* to be relatively unaffected by *any of the* types of stimulation

Conclusions It is *concluded* from the results that the neuronal and the capillary glia exhibit marked enzymic *differences in* a number of respects The reactions of the *neuronal* glia *argue in* favour of a metabolic relation with the nerve cell The enzymic properties of the capillary glia on the other hand reflect functional differences between *it* and the neuronal glia It is suggested that the capillary glia *together with the* vascular endothelium is of importance *in the* transport of material between blood and brain

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The Negative After-Potential of Frog Skeletal Muscle Fibres

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ANDERS PERSSON

TOCKHOLM 1963

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KAROLINSKA INSTITUTET STOCKHOLM 60 SWEDEN

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INTRODUCTION

Intracellular recording has revealed that the action potential of the frog muscle fibre is followed by a negative after potential (Hodgkin and Nastuk 1950) the amplitude and shape of which vary more than the spike potential Desmedt (1953) noted that the membrane potential at the time of the beginning of the after potential is largely independent of the potassium concentration ratio [K]outside/[K]inside across the membrane Since the resting membrane potential depends on the ratio of potassium concentrations it is obvious that the amplitude of the after potential relative to resting potential is also dependent on this ratio The time course of the after potential is said to be exponential (Ishiko and Sato 1956 Frank 1957) and it has been suggested that the membrane is passively recharged through the constant resting leak conductance during the after potential (Ishiko and Sato 1956 Frank 1957 Hutter and Noble 1960) However Macfarlane and Meares (1958*a*) showed that metabolic poisons such as dinitrophenol reduce or even abolish the negative after potential Cooling has a similar effect (Macfarlane and Meares 1958 *b*) Replacement of the chloride ions in the external solution by other halides or by thiocyanate increase both the negative after potential and the twitch tension (Lubin 1957) Findings of this kind suggest that the mechanism underlying the after potential may be more involved than a mere passive recharge of the membrane capacitance through a path of constant permeability

In the present investigation it was found that the membrane conductance was clearly increased during the early part of the after potential and that the membrane potential changed in a rather complicated fashion Later on at times longer than about 15 msec after the peak of the action potential the conductance changes were found to be negligible and the after potential declined with a simple exponential time course The ionic environment was experimentally varied in order to find the relation between the after potential and the ionic currents The effects of dinitrophenol and of repetitive stimulation were also examined

Ivar Hæggstroms Tryckeri AB
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METHODS

The experiments were performed during 1958—1962 on muscles from *Rana temporaria*. Autumn frogs were kept in a cold storage room at 3—5°C and remained in fairly good condition for up to 5—6 months. No obvious seasonal variations were noted in the measured potentials. The sartorius muscle was dissected and mounted horizontally with its deep surface uppermost in a Perspex chamber where it could be viewed with a binocular microscope. The distal tendon and the pelvic insertion were attached to clamps by means of human hairs or fine silk threads. The muscle was stretched to about 1.3 times its length *in situ* in order to reduce movement.

Capillary micro electrodes of less than 0.5 μ tip diameter and filled with 3 M KCl were used for intracellular measurements. Only electrodes with resistances of 10—20 MΩ and with tip potentials less than 5 mV measured in Ringer's solution (Adrian 1956) were used. The micro electrode was connected through an Ag-AgCl electrode to the input cathode follower of a differential DC amplifier (grid current $<10^{-11}$A, input capacity <5 pF). The reference electrode in the external solution was also Ag-AgCl. In some experiments in which chloride ions in the external solution were replaced by methylsulphate, a junction potential appeared making the micro-electrode negative by about 10 mV. This was avoided if the reference electrode was furnished with a KCl bridge. The amplifier was connected to a double beam cathode ray tube.

The stimulating current was supplied by two similar rectangular pulse generators each having an output impedance of 1000 ohms. In most experiments the muscle fibres were excited through a glass pipette having a tip pore of about 100—150 μ diameter and filled with Ringer's solution. Contraction was limited to very few fibres and unnecessary movement minimized if the tip of the electrode was placed quite near the surface of the muscle. Sometimes an intracellular micro electrode was used to pass currents through a single fibre.

The experiments were performed at room temperature (about 20°C). The standard Ringer's solution had the following composition (mM): NaCl 110.0, KCl 2.5, $CaCl_2$ 2.0, $NaHCO_3$ 2.5, Na_2HPO_4 1.2, NaH_2PO_4 0.6, glucose 10, pH 7.3—7.5. Isotonic solutions with different ionic composition were made with the following substitutions: a) KCl was substituted by NaCl, b) Na^+ by choline$^+$, c) Cl^- by $CH_3SO_4^-$, d) NaCl by sucrose. Hypertonic solutions were sometimes used in order to prevent the mechanical contraction (Hodgkin and Horowicz 1957) which often caused a distor

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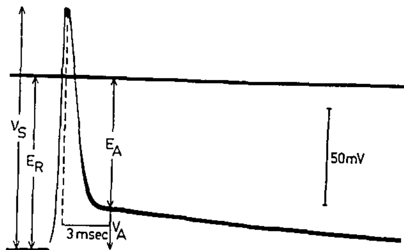


Fig 1 Normal action potential recorded intracellularly The straight line indicates $V = 0$ E_R = resting potential V_s = action potential E_A and V_A = peak amplitude of the negative after potential

tion of the recorded after potentials This distortion was especially disturbing in experiments with methylsulphate because the twitch tension was then approximately twice the normal (Hutter and Noble 1960) These hypertonic solutions were usually made up by adding sucrose and had a tonicity of twice normal A continuous flow of fresh fluid was run in at a rate such that the fluid in the recording cell was changed in about 2 minutes In most experiments a mixture of 5 % carbon dioxide in oxygen was bubbled through the bath solution The degree of oxygen saturation had no detectable effect on the potentials The recording cell held 5 ml solution which could be completely changed in less than one minute Only surface fibres were used and changes in the recorded potentials were observed within two minutes after the beginning of a fluid change In any case the muscles were routinely left for 15 min to permit equilibration with the new solution If hypertonic solutions were tested the muscle was first equilibrated in an isotonic solution and the change to the hypertonic one made just before the penetrations This was done in order to avoid undesirable changes in intracellular ionic composition due to the osmotic effects

Nomenclature Membrane potentials are given as *inside potential minus* outside potential V is used for potentials relative to resting potential and E for absolute values Thus $V = E - E_R$

The following abbreviations are used (see Fig 1) E_R = *resting potential* V_s = action potential E_A and V_A = negative after potential the peak value of E_A and V_A being measured 3 msec after the peak of the action potential τ_A = *the time constant of decay of* V_A

The negative after potential in normal Ringer's solution

Typical action potentials recorded intracellularly are shown in Fig 2 The shape of the beginning of the after potential varied in different muscles and sometimes also among individual fibres in the same muscle Fig 2 shows two examples representing different types of negative after potential The most common type was that in Fig 2 *a* which showed a distinct transition from the falling phase of the spike to the after potential but with $\frac{dI}{dt}$ remaining negative throughout This was found in about two third of 3 900 penetrated muscle fibres from 127 muscles In other muscles $\frac{dI}{dt}$ went through a positive region (Fig 2 *b*) The hump thus appearing reached a maximum about 5 msec after the spike of the action potential The transition between the different forms of the negative after potential was gradual

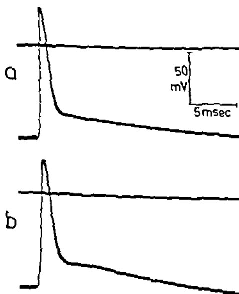


Fig 2 Action potentials showing differences in the shape of the beginning of the negative after potential

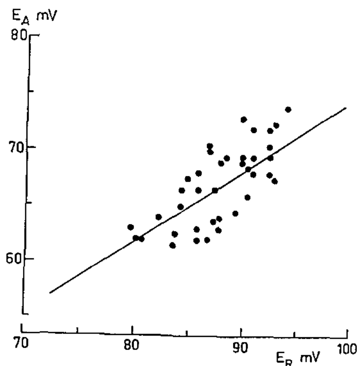


Fig. 3. Peak E_A plotted against E_R. Normal Ringer's solution. The straight line represents $E_A^{peak} = 0·66\,E_R + 9·1$

The peak amplitude of the negative after potential (V_A) was 20·9 ± 3·8 mV (mean and S.D. of 334 fibres from 21 muscles) while the resting potential was 88·0 ± 4·6 mV. These values are in good agreement with previous investigations (Hodgkin and Nastuk 1950; Macfarlane and Meares 1958*a*). The amplitude of the hump, measured as the potential difference between the maximum and minimum values of the after potential, was, as mentioned earlier, quite variable but did not exceed 1 mV in normal Ringer's solution at room temperature.

Fig. 3 is a scatter diagram showing the relation between the peak after potential (E_A) and the resting potential (E_R). The values of E_A and E_R are taken from measurements in normal Ringer's solution and thus represent normal variations. The regression line calculated by the method of least squares had the equation $E_A^{peak} = 0·66\,E_R + 9·1$ in this case. In other experiments the slope of the regression line varied between 0·22 and 0·75. These values agree well with earlier measurements made by Frank (1957).

Desmedt (1953) found in experiments in which [K] and [K] were varied that the peak value of E_A was independent of the concentration changes

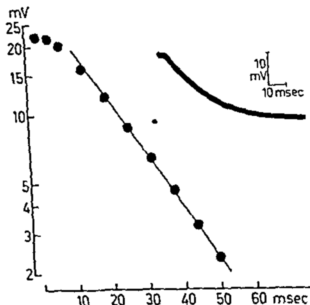


Fig 4 Semilogarithmic plot of the peak amplitude V_A of the after potential against time from the recorded after potential shown in the inset.

and consequently independent of the accompanying changes in resting potential The present findings apparently disagree with those of Desmedt However Desmedt investigated only the effect of increased [K]$_i$ and decreased [K] relative to the normal concentration and consequently only the effect of increased E_R In other experiments (see p 13) the dependence of peak E_A on E_R was further examined when [K] was varied These experiments showed that the peak value of E_A was independent *or only* slightly dependent on E_R in the region where E_R was higher than normal

The time course of the decay of the negative after potential is plotted in Fig 4 The semilogarithmic plot shows that at times longer than about 15 msec the decay followed reasonably well a simple exponential time course At shorter times this was not the case It has been suggested (Frank 1957 Hutter and Noble 1960) that the specific permeability changes which are the cause of the action potential are terminated soon after the spike potential i e that the permeability after the spike is the same as the permeability of the resting membrane The after potential would consequently show nothing more than how the membrane capacitance is discharged

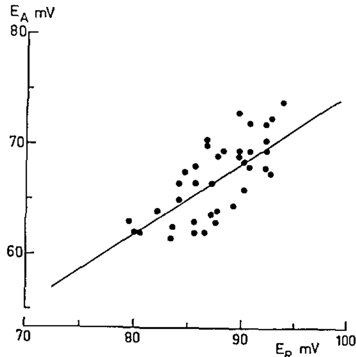


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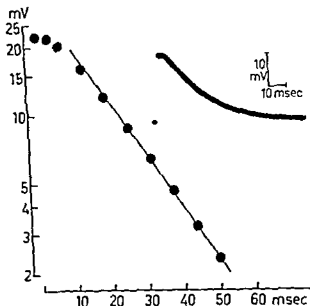


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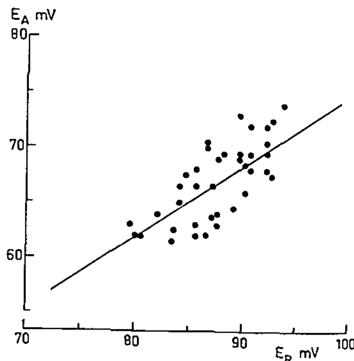


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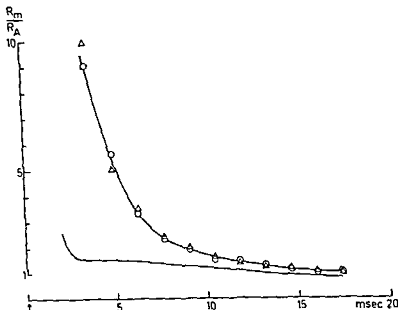


Fig 6 R_m/R_A plotted against time (t = 0 at the peak of the action potential) O = in a normal Ringer's solution △ = in a solution containing 4 m-equiv Cl⁻/l The continuous line drawn through the circles The time course of an after potential is indicated

The effect of constant currents on the negative after potential

In some experiments a small constant current was applied through a micro electrode inserted close to the recording electrode and the effect of this current on the negative after potential was measured If this current is large then it affects the time course of the action potential and the negative after potential since the specific permeability changes depend on membrane potential and time If however the current is made very small then the time courses of the permeability changes will be almost unaffected and the difference between two records one with and one without the extra current will indicate how the membrane resistance changes (cf Hodgkin and Rushton 1946)

Fig 5 shows the records from such an experiment The effect of the constant current on the resting membrane was first measured Then two successive action potentials were elicited by two brief shocks one without and one during the application of a constant anodal current A third action potential was then recorded without a constant current The three

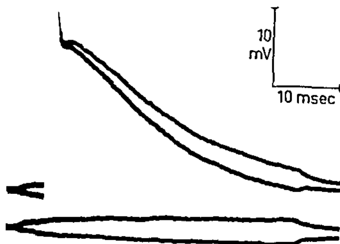


Fig 5 Above *superimposed after potentials during constant anodal and cathodal currents* Below membrane potential change associated with a step of injected current Calibration 10 mV

through a *channel that has a constant* permeability equal to the resting permeability The present findings indicate that this might be the case at times longer than about 15 *msec, while at shorter times the situation is* clearly more complicated The time constant (τ_A) of the decay of the after potential ($t > 15$ msec) was 19 8 ± 6 1 msec (mean and S D) in 111 fibres from 17 muscles The time constant of the resting membrane (τ_m) was 18 2 ± 4 3 msec (mean and S D) This latter value was obtained from measurements on 78 of the same fibres on which τ_A was determined τ_m was measured from the decay of membrane potential towards its resting value from its value immediately after a small step of anodal current was applied through another micro electrode inserted about 50 μ from the recording electrode (see Fatt and Katz *1951*) τ_A and τ_m *did not significantly* deviate from each other in the present experiments These findings are *therefore consistent* with the interpretation of Frank (1957) and of Hutter and Noble (1960) noted earlier if this interpretation is restricted to only the late part of the after potential However unless it is shown that the membrane resistance during the after potential has the same value as that of the resting membrane there is still the possibility that the similarity between the values of τ_A and τ_m is a mere coincidence *The* divergence of the decay of peak I A from a simple exponential curve at short times might indicate that the *membrane permeability changes* during the beginning of the after potential

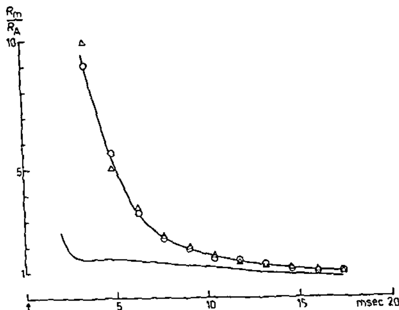


Fig 6 R_m/R_A plotted against time (t = 0 at the peak of the action potential) ○ = in a normal Ringer's solution △ = in a solution containing 4 m-equiv Cl^-/l The continuous line drawn through the circles The time course of an after potential is indicated

The effect of constant currents on the negative after potential

In some experiments a small constant current was applied through a micro-electrode inserted close to the recording electrode and the effect of this current on the negative after potential was measured If this current is large then it affects the time course of the action potential and the negative after potential since the specific permeability changes depend on membrane potential and time If however the current is made very small then the time courses of the permeability changes will be *almost* unaffected and the difference between two records one with and one without the extra current will indicate how the membrane resistance changes (cf Hodgkin and Rushton 1946)

Fig 5 shows the records from such an experiment The effect of the constant current on the resting membrane was first measured Then two successive action potentials were elicited by two brief shocks *one* without and one during the application of a constant anodal current A third action potential was then *recorded* without a constant current The three

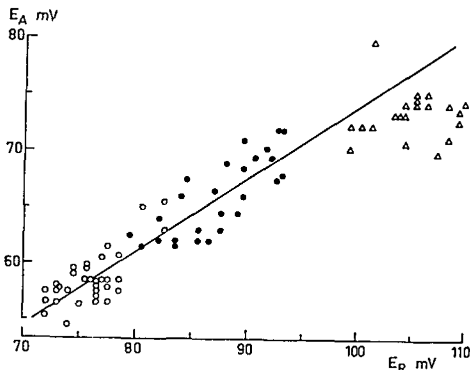


Fig 7 E_A plotted against E_R in solutions containing 0.5 (△) 2.5 (●) and 5.0 (O) m equiv K^+/l The regression line satisfies $E_A^{peak} = 0.67\,E_R + 8.4$

records were taken on the same frame If the first and third deviated from each other the measurements were discarded High amplification was used in order to obtain resolution with currents as weak as possible Mechanical *injury during the contraction was held to be the major cause of the* changes between the first and third action potentials in a run The percentage of successful runs was also larger in experiments in which mechanical movement was diminished by hypertonic solutions (cf Hodgkin and Horowicz 1957)

In Fig 6 the ratio between resting membrane resistance and resistance during the after potential (R_m/R_A) is plotted against time as measured from the peak of the action potential The time course of an action potential is also indicated It is seen that the membrane resistance at the beginning of the negative after potential was about a tenth of that of the resting membrane and then gradually increased although it was still lower than the resting value 10 msec after the peak of the action potential At the latter time the after potential amplitude was about half its initial value At times longer than about 15 msec the membrane resistance was close to its resting value while the membrane potential clearly had not yet reached its resting value (Ishiko and Sato 1956 Persson 1960)

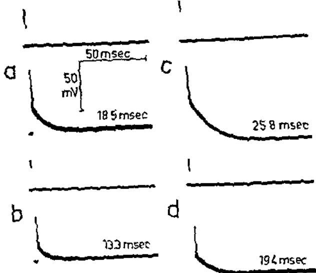


Fig 8 Records from muscle fibres in various potassium concentrations a) and d) 2.5 m-equiv K+/l b) 5.0 m-equiv K+/l c) 0.5 m-equiv K+/l

The effect of changes in external potassium concentration

The measurements described earlier (p 8) were made on muscle fibres in a normal ionic environment Experiments were also made in which the ionic composition of the external solution was varied

The peak value of E_A was observed to depend on the resting potential (Fig 3) Since the resting potential depends on the ratio [K]/[K] (Boyle and Conway 1941 Adrian 1956 Hodgkin and Horowicz 1959) it seemed logical to extend the measurements of the after potential to solutions containing different potassium concentrations In Fig 7 the peak value of E_A is plotted against resting potential for different values of $[K]_o$ (5.0 2.5 and 0.5 mM) The peak E_A was clearly dependent on the resting potential when the latter was less than about 100 mV but only little affected or independent at resting potentials above 100 mV This may be compared with the results from the experiments made by Benoit and Coraboeuf (1955) where it is shown that peak E_A was not affected by changes in the resting membrane caused by anodal or cathodal currents The time constant of the decay of the negative after potential was 13.8 ± 0.95 in 5 mM KCl and 27.6 ± 0.84 msec in 0.5 mM KCl (Fig 8) This change in the time constant τ_A is of the same order of magnitude as the change in the

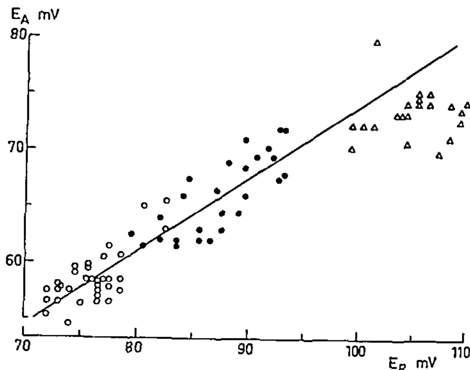


Fig 7 E_A plotted against E_R in solutions containing 0.5 (△) 2.5 (●) and 5.0 (○) m equiv K^+/l The regression line satisfies $E_A^{peak} = 0.67\,E_R + 8.4$

records were taken on the same frame If the first and third deviated from each other, the measurements were discarded High amplification was used in order to obtain resolution with currents as weak as possible Mechanical injury during the contraction was held to be the major cause of the changes between the first and third action potentials in a run The percentage of successful runs was also larger in experiments in which mechanical movement was diminished by hypertonic solutions (cf Hodgkin and Horowicz 1957)

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equilibrium had been reached and showed no shift in either resting membrane potential (cf Hodgkin and Horowicz 1959) or in negative after potential peak E_A. When $[Cl]$ was reduced however the after potential consistently had a notch with a maximum amplitude occurring 5—6 msec after the peak of the action potential. The amplitude of the notch measured as the difference between the maximum and the preceding minimum of the membrane potential increased at decreased $[Cl]_o$. The notch was 2.44 ± 0.59 mV in a solution containing 59 m equiv Cl^-/l (33 observations) and 3.71 ± 0.62 mV in 6.5 m equiv Cl^-/l (41 observations). The relation between peak E_A and E_R was unaffected by alterations of $[Cl]_o$. As shown previously (p 13) peak E_A was dependent on E_R when E_R was less than about 100 mV.

In other experiments $[Cl]_o$ was increased to 1.5 or 2 times the normal by adding equivalent amounts of choline chloride. The sodium concentration remained constant in these solutions. The resting membrane potential and negative after potential peak E_A were unaffected. It was observed however that the negative after potential never began with a plateau or notch in solutions having increased $[Cl]_o$.

Measurements of the membrane resistance were made (p 11) with the muscle in solutions in which $[Cl]$ was reduced by replacement with $CH_3SO_4^-$ or I^-. The results from such an experiment are shown in Fig 6. In this experiment all but 4 m equiv Cl^-/l was replaced by methylsulphate. It was obvious that the membrane resistance change *during* the after potential remained unaffected by the change in $[Cl]$.

The time constant of the decay of the after potential was however affected by changes in $[Cl]$, i e τ_A increased when $[Cl]_o$ was reduced (Table I). Such a change of τ_A agrees with the results of Hutter and Noble (1960).

Table I The effect of changes in $[Na]$ and $[Cl]$ (means and S D)

$[Na]$/$[Cl]$ m-equiv/l	E_R mV	V mV	E_A mV	Ampl of notch mV	τ_A msec
116.5/118.5	88.0 ± 4.6	129	65.1 ± 5.2	0	19.8 ± 6.1
240 /118.5	89.7 ± 5.3	139	64.2 ± 4.9	1.55 ± 0.65	23.2 ± 7.0
240 / 59	93.3 ± 5.4	140	69.7 ± 5.4	3.3 ± 0.53	27.5 ± 6.6
116.5/ 59	97.6 ± 6.1	126	68.4 ± 4.8	2.44 ± 0.59	40.2 ± 6.5
116.5/ 6.5	94.3 ± 5.9	132	70.0 ± 5.1	3.71 ± 0.62	41.8 ± 8.1
58 / 59	87.6 ± 4.2	103	64.8 ± 4.8	0	18.5 ± 4.6
240 / 240	89.5 ± 5.1	141	65.2 ± 6.2	0	20.4 ± 5.8

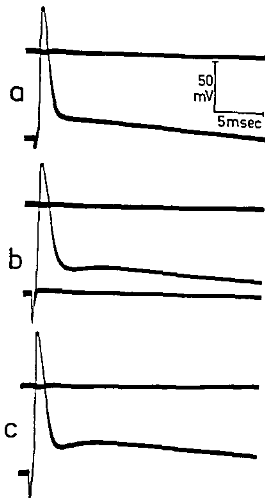


Fig. 9. Action potentials in solutions containing different chloride concentrations. a) 118.5 m-equiv/l. b) 59 m-equiv/l. c) 6.5 m-equiv/l.

time constant of the resting membrane for corresponding changes in the resting potential (Jenerick 1953).

The effect of changes in external chloride concentration [Cl]

In a paper by Hutter and Noble (1960) it is pointed out that Cl^- carries a large part of the current during the repolarization. Changes in [Cl] also alter the shape of the negative after-potential, especially its beginning. These effects are most pronounced if Cl^- is replaced by an anion to which the muscle membrane is impermeable. Experiments were made in which chloride was replaced by methylsulphate to various extents. Sodium and potassium concentrations were in all these experiments kept constant. Fig. 9 shows action potentials recorded from a fibre in solutions having different external chloride concentrations. The records were taken after

equilibrium had been reached and showed no shift in either resting membrane potential (cf Hodgkin and Horowicz 1959) or in negative after potential peak E_A. When $[Cl]_o$ was reduced however the after potential consistently had a notch with a maximum amplitude occurring 5—6 msec after the peak of the action potential. The amplitude of the notch measured as the difference between the maximum and the preceding minimum of the membrane potential increased at decreased $[Cl]_o$. The notch was 2.44 ± 0.59 mV in a solution containing 59 m-equiv Cl^-/l (35 observations) and 3.71 ± 0.62 mV in 6.5 m-equiv Cl^-/l (41 observations). The relation between peak E_A and E_R was unaffected by alterations of $[Cl]_o$. As shown previously (p. 13) peak E_A was dependent on E_R when E_R was less than about 100 mV.

In other experiments [Cl] was increased to 1.5 or 2 times the normal by adding equivalent amounts of choline chloride. The sodium concentration remained constant in these solutions. The resting membrane potential and negative after potential peak E_A were unaffected. It was observed however that the negative after potential never began with a plateau or notch in solutions having increased [Cl].

Measurements of the membrane resistance were made (p. 11) with the muscle in solutions in which [Cl] was reduced by replacement with $CH_3SO_4^-$ or I^-. The results from such an experiment are shown in Fig. 6. In this experiment all but 4 m-equiv Cl^-/l was replaced by methylsulphate. It was obvious that the membrane resistance change during the after potential remained unaffected by the change in [Cl].

The time constant of the decay of the after potential was however affected by changes in [Cl], i.e. τ_A increased when [Cl] was reduced (Table I). Such a change of τ_A agrees with the results of Hutter and Noble (1960).

Table I The effect of changes in [Na] and [Cl] (means and S.D.)

$[Na]_o/[Cl]$ m-equiv/l	E_R mV	V_s mV	E_A mV	Ampl. of notch mV	τ_A msec
116.5/118.5	88.0 ± 4.6	129	65.1 ± 5.2	0	19.8 ± 6.1
240 /118.5	89.7 ± 5.3	139	64.2 ± 4.9	1.55 ± 0.65	23.2 ± 7.0
240 / 59	9[illegible].3 ± 5.4	140	69.7 ± 5.4	3.3 ± 0.55	27.5 ± 6.6
116.5/ 59	9[illegible].6 ± 6.1	1[illegible]	68.4 ± 4.8	2.44 ± 0.59	30.2 ± 6.5
116.5/ 6.5	94.3 ± 5.9	132	70.0 ± 5.1	3.71 ± 0.62	41.8 ± 8.1
58 / 59	87.6 ± 4.2	103	64.8 ± 4.8	0	18.5 ± 4.6
240 /240	85.5 ± 5.1	141	65.2 ± 6.2	0	20.4 ± 5.8

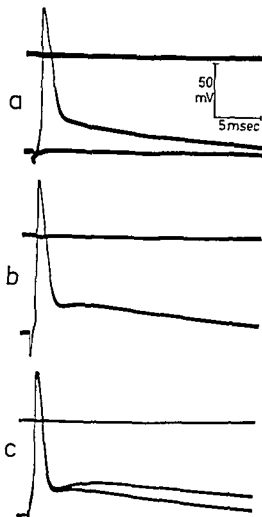


Fig 10 Action potentials in solutions containing a) normal $[Na]_o$ and $[Cl]_o$ b) 240 m equiv/l $[Na]_o$ and 118 5 m-equiv/l $[Cl]_o$ c) 240 m-equiv/l $[Na]_o$ 118 5 m-equiv/l $[Cl]_o$ and 240 m-equiv/l $[Na]_o$ 59 m-equiv/l $[Cl]_o$ superimposed

The effect of changes in external sodium concentration $[Na]_o$

The sodium concentration was varied in some experiments while [Cl] and [K] were kept constant In sodium deficient solutions sodium chloride was replaced by choline chloride in sodium rich solutions sodium methyl sulphate or glutamate were added Reduction of [Na] to 58 or 33 m equiv/l had no detectable effect on the peak amplitude of the after potential although a minute effect on the shape of the after potential was observed The after potential never began with a notch or plateau when the

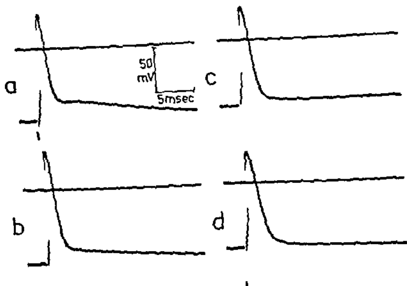


Fig 11 Action potentials after various times in a Ringer's solution containing 0.5 mM DNP a) 2 min b) 7 min c) 15 min d) 30 min

fibre was in sodium-deficient solutions When, on the *other hand* the sodium concentration was increased from 112 to 224 m-equiv/l., the after potential began regularly with a notch or a plateau (Fig 10) (Persson 1962) The notch reached its maximum 5 msec after the peak of the action potential with an amplitude *of* 1.55 ± 0.63 mV (mean and S.D.) The time constant τ_A was *as a rule only* little affected by the change in sodium concentration but *sometimes* τ_A *increased* slightly in high [Na] (cf Frank 1957) The peak after potential E_A was not significantly changed in high $[Na]_o$ The change in peak amplitude *of* the *action* potential was again near to that predicted by *Nernst's equation* as pointed out by Nastuk and Hodgkin (1950) It might be *mentioned that* in solutions with low $[Cl]_o$ and high [Na] a notch of considerable *amplitude* (4—5 mV) was observed about 5 msec after the peak *of the* action potential (Fig 10) In some instances the membrane currents *were regenerative* so that a second action potential started at this time This was especially the case in calcium deficient solutions

The finding that a second action potential *could* take off from the notch on the after potential in solutions containing high [Na] and low [Ca] *clearly*

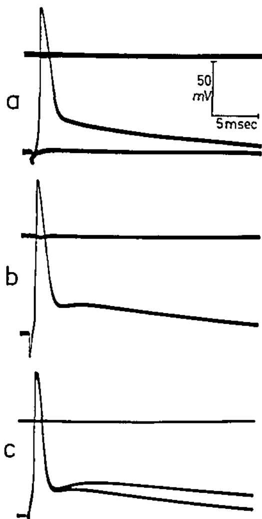


Fig 10 Action potentials in solutions containing a) normal $[Na]_o$ and $[Cl]_o$ b) 240 m-equiv/l $[Na]_o$ and 118.5 m-equiv/l $[Cl]_o$ c) 240 m-equiv/l $[Na]_o$ 118.5 m-equiv/l $[Cl]_o$ and 240 m-equiv/l $[Na]_o$ 59 m-equiv/l $[Cl]_o$ superimposed

The effect of changes in external sodium concentration $[Na]_o$

The sodium concentration was varied in some experiments while [Cl] and [K] were kept constant In sodium deficient solutions sodium chloride was replaced by choline chloride in sodium rich solutions sodium methyl sulphate or glutamate were added Reduction of [Na] to 58 or 33 m-equiv/l had no detectable effect on the peak amplitude of the after potential although a minute effect on the shape of the after potential was observed The after potential never began with a notch or plateau when the

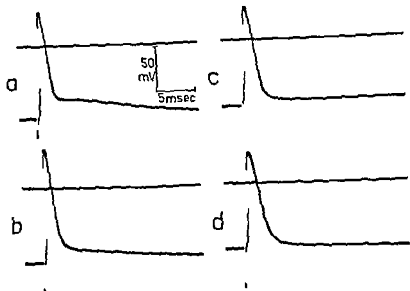


Fig 11 Action potentials after various times in a Ringer's solution containing 0 5 mM DNP a) ? min b) 7 min c) 15 min d) 30 min

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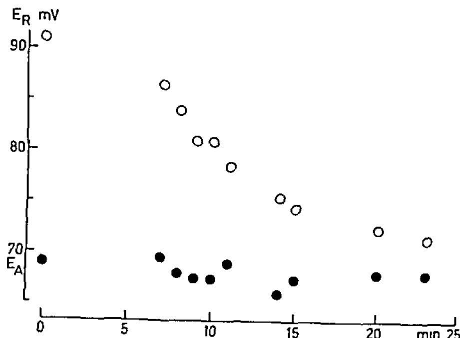


Fig. 12 E_R (O) and E_A (●) plotted against time in a Ringer's solution containing 0.5 mM DNP

indicates that the after potential was across the excitable membrane and not across some other structure in series with the excitable membrane

The effects of $[Na]_o$ and $[Cl]_o$ are summarized in Table I

The effect of dinitrophenol

The action of 2 4 dinitrophenol and other metabolic inhibitors on the peak amplitude of the negative after potential has been studied in detail by Macfarlane and Meares (1958*a*) They found that the amplitude of the after potential (V_A) decreased if the concentration of the inhibitor was large enough and the time of action on the muscle was sufficiently long These results were confirmed in the present investigation Fig 11 shows action potentials at various times after a Ringer's solution containing 0 5 mM 2 4 dinitrophenol was applied The peak amplitude of the negative after potential (V_A) was after 15 minutes reduced to about half its initial value and after 30 minutes it was nearly zero The amplitude of the action potential had decreased by 10—15 mV at 30 minutes and the slope of the falling phase had decreased from 150 V/sec to about 60 V/sec The plot

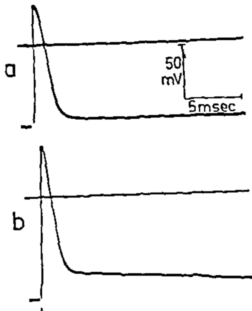


Fig. 13 a) Action potentials after 5 min in a 0.5 mM DNP Ringer's solution b) the same muscle fibre after $[K]_o$ was reduced to 0.5 m-equiv/l

in Fig. 12 shows that the resting potential decreased by about 20—25 mV but the value of the membrane potential at the time of the beginning of the after potential (E_A) was but little affected. This clearly appears as a decline of the peak value of V_A. Note that an increase in $[K]_o$ decreased E_A nearly as much as E_R (see p. 13). If the resting potential was increased in a DNP poisoned fibre the negative after potential reappeared. Fig. 13 shows action potentials from such an experiment. V_A was small in normal $[K]$ and DNP and clearly increased in the solution containing 0.5 m-equiv K^+/l and DNP. There was no significant change of E_A.

The time constant of the resting membrane was measured in solutions with and without 0.5 mM DNP as previously described (p. 10). τ_m decreased to about half its original value in 30 minutes (19.2 ± 5.9 msec in normal Ringer's solution and 10.8 ± 5.1 msec in the solution containing DNP). The time constant τ_A of the decay of the after potential was however not measurably affected by DNP. This finding contrasted with the effect of altered $[K]_o$ because changes in $[K]$ affected τ_m and τ_A about equally.

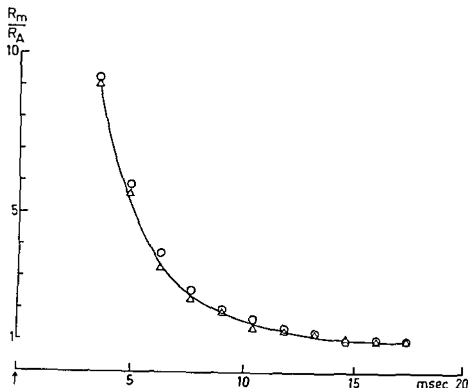


Fig 14 R_m/R_A *plotted against time after 30 min in a Ringer's solution without (△) and* with 0 5 mM DNP (O) The continuous line drawn through the triangles

The membrane resistance during the time of the after potential was *also measured on DNP* poisoned fibres (see p 11) These measurements (Fig 14) indicated that the change in membrane resistance during the after potential was only negligibly affected by DNP (Persson 1960)

The effect of repetitive stimulation

In the previous section it was shown that a metabolic inhibitor such as dinitrophenol clearly affected the negative after potential In these experiments is was regularly noted that the contractions of the muscle fibre became weaker during the action of *DNP* The findings *of Loomis and Lipmann* (1948) and Green (1951) that DNP reduces available high energy phosphates might explain the effect on the contractions Twitch tension *also declines progressively during* repetitive activity (fatigue) It is known that in prolonged muscular activity energy is consumed from the break down of creatine phosphate (CrP) and *probably adenosine triphosphate*

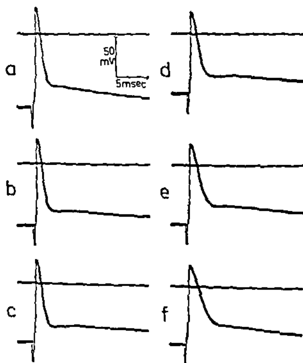


Fig 1 Action potentials recorded at 15 second intervals during repetitive stimulation at 10 *impulses/sec*

(ATP) The immediate source of energy for a single contraction however is still unknown (Davies and Cain 1961) If lack of metabolic energy is the cause of the reduction in the peak amplitude of the negative after potential in a DNP Ringer's solution then similar changes in the action potential and the negative after potential might appear progressively during repetitive activity

Muscle fibres were stimulated repetitively for some minutes at a rate of 3 to 30 impulses per second The membrane potential was as a rule recorded continuously through a micro electrode while the stimuli were applied through extracellular electrodes Fig 1a shows typical action potential recorded at 15-second intervals during a 75-second period of repetitive stimulation at 10/sec Small progressive decreases of both resting potential and overshoot of the spike were observed Resting potential declined some 5 to 10 mV after such stimulation The rate of decline was greater at

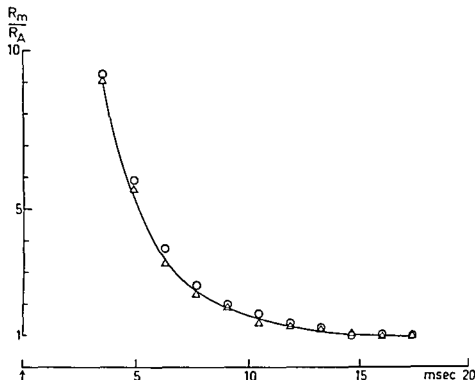


Fig 14 R_m/R_A plotted against time after 30 min in a Ringer's solution without (△) and with 0.5 mM DNP (○) The continuous line drawn through the triangles

The membrane resistance during the time of the after potential was also measured on DNP poisoned fibres (see p 11) These measurements (Fig 14) indicated that the change in membrane resistance during the after potential was only negligibly affected by DNP (Persson 1960)

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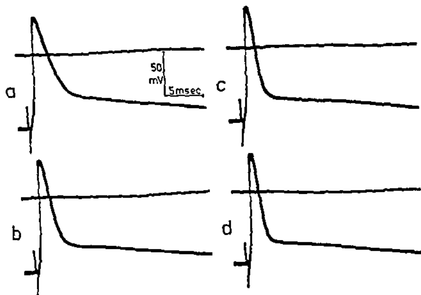


Fig 17 Recovery after 90-sec period of repetitive activity at a stimulation rate of 10 per sec a) fibre impaled just after a 90-sec period of stimulation b) c) d) recorded 1 3 and 6 min respectively after a

activity The most obvious changes in the action potential during repetitive activity were a decrease in the maximum rate of decline of the action potential and an increase in the peak amplitude of the negative after potential (V_A) There was also a decrease in F_A In Fig 16 the maximum rate of decline of the action potential is plotted against the number of preceding impulses Both the maximum rate of decline and V_A changed continuously with the number of impulses These changes were larger but otherwise similar at higher rates of stimulation (Fig 16) In most of the experiments of this kind the rate of stimulation was 10/sec at other rates the experimental changes were less reversible and the mechanical damage probably greater Results obtained from a number of experiments with various rates of stimulation are summarized in Table II

In addition to the effect of repetitive stimulation on the maximum rate of decline of the action potential and V_A there was a decrease in the time constant of the decay of the after potential τ_A with the number of preceding impulses as described previously by Ishiko and Sato (1956) and Osborne (1958) The change in τ_A was however more variable than the changes in the maximum rate of decline and V_A

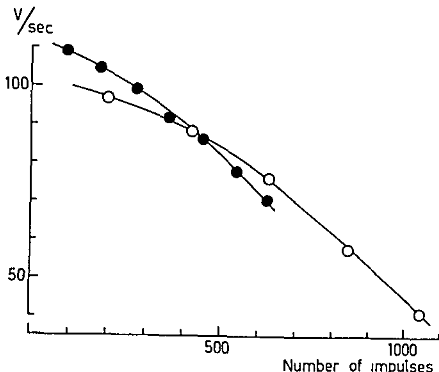


Fig 16 Maximum rate of fall of the action potential plotted against the number of preceding impulses at two different stimulation rates ● at 5 impulses/sec ○ at 15 impulses/sec

the beginning of the stimulation period Some mechanical damage to the membrane caused by the micro electrode can not be excluded in these experiments, in which the membrane was penetrated before the period of

Table II The effect of repetitive activity at different rates of stimulation after a 60 second period of stimulation (means)

Rate of stim impulses/sec	$E_R - \bar{E}_R$ mV	$V_A - \bar{V}_A$ mV	Max rate of fall V/sec	$\tau_A - \bar{\tau}_A$ msec
5	10	4	90	5.8
10	6	7	76	8.1
15	8.5	12.7	58	7.2

$\bar{E}_R$ $\bar{V}_A$ $\bar{\tau}_A$ indicate the values of E_R V_A and τ_A respectively after a 60 second period of stimulation

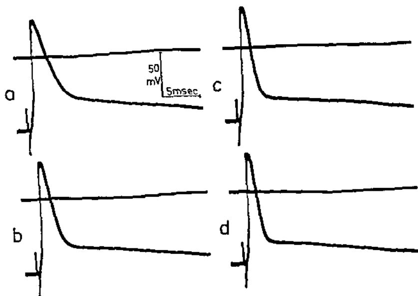


Fig 1 Recovery after 90-sec period of repetitive activity at a stimulation rate of 10 per sec a) Fibre impaled just after a 90-sec period of stimulation b) c) d) recorded 1 3 and 6 min respectively after a

activity The most obvious changes in the action potential during repetitive activity were a decrease in the maximum rate of decline of the action potential and an increase in the peak amplitude of the negative after potential (V_A) There was also a decrease in E_A In Fig 16 the maximum rate of decline of the action potential is plotted against the number of preceding impulses Both the maximum rate of decline and V_A changed continuously with the number of impulses These changes were larger but otherwise similar at higher rates of stimulation (Fig 16) In most of the experiments of this kind the rate of stimulation was 10/sec at other rates the experimental changes were less reversible and the mechanical damage probably greater Results obtained from a number of experiments with various rates of stimulation are summarized in Table II

In addition to the effect of repetitive stimulation on the maximum rate of decline of the action potential and V_A there was a decrease in the time constant of the decay of the after potential τ_A with the number of preceding impulses as described previously by Ishiko and Sato (1956) and Osborne (1958) The change in τ_A was however more variable than the changes in the maximum rate of decline and V_A

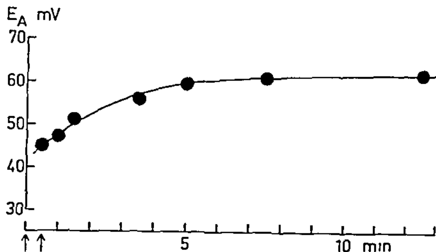


Fig 18 E_A plotted against time after a 90 sec period of activity The arrows indicate the beginning and end of repetitive activity

The described alterations in the maximum rate of decline of the action potential and V_A were reversible in several experiments but only partially so in others How far membrane damage due to the *penetrating electrode* affected this reversibility was impossible to judge in these experiments Other experiments were therefore made in which the muscle fibre was activated in a similar way for a period of 90 sec at a rate of 10/sec before the recording electrode was introduced into the fibre (Fig 17) In such a case the contraction of the fibre was weak and the damage caused by the electrode much smaller The effects of repetitive activity on the resting potential action potential and after potential were qualitatively the same as in the other experiments The change in resting potential was a few millivolts less The time course of the recovery measured from single impulses every 30 seconds was found to be 50 % complete in about 3 minutes In Fig 18 E_A is plotted against time after the period of activity

Changes in the external ionic environment seemed to have very little *influence on the effect of repetitive activity on the action* potential and the negative after potential However, when part of the chloride in the Ringer s solution was replaced by methylsulphate the decrease in the maximum rate of decline of the action potential and the increase in V_A occurred more slowly than in Ringer s solution at the same rate of stimulation Alteration of neither the sodium nor the potassium concentration in the Ringer s solution had a similar effect

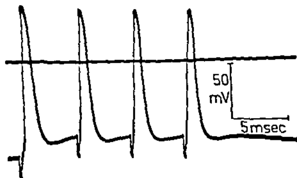


Fig 19 Four action potentials elicited in quick succession Normal Ringer's solution

Fig 19 shows the record from another type of experiment Four action potentials were elicited in quick succession the three latest appearing during the time of the after potential of the first action potential It is clearly seen that the amplitudes of the second and subsequent after potentials (*F* x) were entirely unaffected by the first action potential

DISCUSSION

It has previously been shown that the peak amplitude of the negative after potential is affected by various factors that are known to interfere with muscle metabolism Findings of this kind might indicate that the after potential is caused by membrane currents of a metabolic origin (Macfarlane and Meares 1958 *a*) In the present experiments it was found that the effect of DNP was mainly to reduce the resting potential (E_R) without affecting the membrane potential at the time of the beginning of the after potential (E_A) Furthermore, the after potential had a normal shape when the solution containing DNP was deficient in KCl i e when the depolarizing effect of DNP was counteracted by the change in KCl concentration (Fig 13) Moreover, it was shown that the membrane resistance was reduced at the time of the beginning of the after potential and that this reduction in membrane resistance was about the same as occurred in solutions with DNP These findings indicate that the effect of DNP was mainly on the resting membrane currents and that the membrane permeability changes during the after potential were largely unaffected by DNP Therefore it cannot be concluded that the after potential is caused by metabolically generated currents

The studies of Loomis and Lipmann (1948) and Green (1951) have shown that DNP uncouples oxidation from phosphorylation and reduces available high energy phosphates In the squid nerve it has been shown by Hodgkin and Keynes (1955) that the presence of DNP in low concentrations (0 5—2 mM) increases the net loss of intracellular potassium and that active absorption of potassium is inhibited by DNP Keynes and Maisel (1954) found that the resting potential of frog muscle decreases somewhat when the muscle is poisoned by DNP (0 2 mM) while the intracellular potassium concentration was found to be unaffected by DNP Contrary to this Shaw and Simon (1955) found that the intracellular potassium concentration was reduced after poisoning with DNP Since Shaw and Simon used a higher concentration of DNP (1 mM) it is possible that the difference in concentration might explain the discrepancy in results Calculations indicate that $[K]_i$ would be reduced to about half its normal value in a poisoned fibre with a resting potential of 75 mV if the change in resting potential were due entirely to a change in $[K]_i$ So large a change in $[K]_i$ seems unlikely The reason why the resting potential is decreased in a DNP poisoned fibre thus remains obscure

One possibility is that the negative after potential in the muscle fibre develops in a manner similar to the negative after potential in the squid giant nerve fibre (Frankenhaeuser and Hodgkin 1956) i e the potassium concentration in a small limited space outside the active membrane increases during the action potential as a result of the increased net outward flux of potassium (cf Adrian and Freygang 1962) If this were the case the after potentials of successive impulses ought to be added to each other The experiments described on p 25 in which a few action potentials were rapidly elicited indicate that the amplitude of the second and subsequent after potentials were entirely unaffected by the first action potential This finding is inconsistent with the hypothesis that the negative after potential is dependent on potassium ions accumulated in a limited space outside the active membrane

It has been suggested that the specific permeability changes which are the cause of the action potential have ceased at the time of the beginning of the after potential (Falk 1961) The after potential would then only indicate the passive recharge of the membrane capacitance through the permeability channel of the resting fibre The measurements of the membrane resistance during the after potential however indicated that the permeability changes were still appreciable 10 msec after the peak of the action potential i e during at least a large fraction of the after potential This is further supported by the fact that the time course of the first half of the after potential is not simply exponential

The present experiments indicate therefore that the negative after potential is closely associated with permeability changes in the membrane and that these permeability changes are but little affected by the metabolic inhibitor DNP For the present it seems likely that as suggested by Doctor B Frankenhaeuser a small late increase in sodium permeability or a small late increase in a non specific permeability similar to that described by Frankenhaeuser (1962) in the toad nerve fibre might explain most of the properties of the negative after potential The relatively small effect of changes in [Na] and [Cl] are clearly consistent with such a hypothesis The experiments in which two action potentials were elicited close to each other in time (Fig 19) are also interesting from another point of view The second action potential started from a lower value of membrane potential than the first, 67 mV compared to 87 mV From experiments in which E_A was related to resting potential (Fig 3) it would be expected that the peak amplitude of E_A would change by about 10—15 mV if it were the membrane potential preceding the action potential which

affected and determined the peak amplitude of the after potential The peak value of E_A was, however, unaffected This would be expected if it is assumed that it was the current during the after potential that modified the after potential and not the membrane potential preceding the action potential The large change in peak E_A when $[K]_i$ was increased and resting membrane potential lowered and the absence of changes of E_A when $[K]_i$ was low would be explained in a simple and straightforward manner the small change in potassium current (I_K) at low potassium concentrations is insufficient to affect the membrane potential when the membrane has a low impedance at the time of the after potential but is sufficient to affect the resting membrane which has a much higher impedance

The specific permeability changes which occur in the muscle fibre are not yet sufficiently understood to permit a quantitative explanation of the after potential Further information is therefore needed

SUMMARY

The negative after potential of frog skeletal muscle fibres has been studied with the micro electrode technique under various experimental conditions.

It was found that the peak amplitude of the after potential (E_A) was dependent on the resting potential (E_R) when the latter was less than 100 mV. For E_R greater than 100 mV, E_A was nearly independent of E_R.

Changes in the external sodium or chloride concentrations had a relatively small effect on the amplitude and shape of the beginning of the after potential.

At times longer than about 15 msec after the peak of the action potential the decay of the after potential was exponential. At shorter times the membrane potential behaved in a more complicated manner.

During the first 15 msec of the after potential the membrane resistance was decreased.

The effect of DNP on the resting potential and after potential was investigated. While DNP decreased the resting potential, the membrane potential at the time of the beginning of the after potential was nearly unchanged.

The after potentials of action potentials elicited in quick succession were not added to each other. During repetitive activity the peak amplitude of the after potential (E_A) decreased progressively, while the resting potential was only little affected.

The experiments indicate that the negative after potential is closely associated with permeability changes in the membrane and that these permeability changes are but little affected by the metabolic inhibitor DNP. A small late increase in sodium permeability or in a non specific permeability may explain most of the properties of the negative after potential.

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KAROLINSKA INSTITUTET
STOCKHOLM 60 SWEDEN

CATECHOLAMINES AND 5-HYDROXYTRYPTAMINE IN MORPHINE TOLERANCE AND WITHDRAWAL

BY

LARS M GUNNE

STOCKHOLM 1963

CONTENTS

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TRYCKERI AKTIEBOLAGET THULE

INTRODUCTION

It is an old clinical observation that morphine addicts can withstand large amounts of the drug without showing conspicuous manifestations of physical or mental alteration. *Withdrawal of morphine* however precipitates the characteristic abstinence syndrome, which shows many features indicative of a gross disturbance of the autonomic nervous system, together with excitation which is sometimes exaggerated to the point of delirium. After a few stormy days the recovery process becomes evident and the characteristic syndrome generally subsides in about ten days.

KOLB and HIMMELSBACH (1938) have developed a quantitative method for the scoring of all measurable abstinence manifestations in man. Some of these signs and symptoms are: restlessness, rise in respiratory rate and pulse rate, rise in blood pressure, fever and rise in basal metabolism, rise in blood sugar, dilated pupils, tremor, pilo-erection, reduced weight and calory intake, insomnia, yawning, rhinorrhea, lacrimation, perspiration, emesis and diarrhea.

An important contribution to the study of abstinence phenomena was offered by the observation of ISBELL and FRASER (1950) that a complete but transient *abstinence syndrome* was produced in morphine-dependent addicts by injection of N allylnormorphine (nalorphine), thus unmasking the patients' dependence on the drug.

Corresponding symptoms of excitation with several autonomic manifestations have been described in dogs and in some rat strains after withdrawal from long term administration of morphine (JOEL and ETTINGER 1926, PLANT and PIERCE 1928).

The vegetative excitation syndrome is a well recognized phenomenon in brain stimulation and ablation studies on various experimental animals. Such experiments have indicated the importance of limbic and hypothalamic brain areas in modulating autonomic and somatic activity during behavioral reactions (for references see KAADA 1951, HESS 1954).

The discovery of a series of biologically active substances in those areas of the brain stem[1] where autonomic functions had been localized stimulated the rapid evolution of neuropharmacology during the last decade. Soon after the localization of high concentrations of noradrenaline (VOGT 1954) and 5 hydroxytryptamine (serotonin) (AMIN, CRAWFORD and GADDUM

[1] In this paper the brain stem is defined as medulla oblongata, pons, midbrain and diencephalon.

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1954) to certain areas of the brain stem, the discovery was made that the brain content of these monoamines could be influenced by various drugs. The dramatic changes produced by tranquilizers of the reserpine group and the centrally stimulating monoamine oxidase inhibitors have been extensively studied during the last few years, notably with regard to their implications for the function of brain monoamines (for references see BRODIE, SPECTOR and SHORE 1959 a).

The present investigation was prompted by the findings of VOGT (1954) that a single injection of morphine elicits excitation and an increased sympathetic activity in cats, together with a depletion of noradrenaline from the hypothalamic and midbrain stores. These acute effects of morphine in cats actualized the related problem of morphine abstinence and the possible neurochemical mechanisms underlying this syndrome. A series of reports dealing with this problem has appeared earlier from this laboratory (GUNNE 1959, 1960, 1961, 1962 a, b), the present paper is an extension of these earlier studies. It was carried out according to the following general plan.

At first a study was made of the effects of acute and chronic morphine administration, as well as of withdrawal and nalorphine induced abstinence on the general behavior of some laboratory animals. Special attention was paid to species differences observed between dogs, rats and cats (Chapter III). A study was then made of the effects of chronic morphine treatment and abstinence on the brain content of noradrenaline, dopamine and 5-hydroxytryptamine (Chapter IV) and on the adrenal gland content of adrenaline and noradrenaline (Chapter V). In order to obtain additional information concerning the rate of synthesis and liberation of the monoamines, a similar study was finally made of the urinary output of adrenaline, noradrenaline and 5-hydroxyindoleacetic acid (5-HIAA), the main urinary metabolite of 5-hydroxytryptamine (Chapter VI).

CHAPTER I

EARLIER LITERATURE

Occurrence of catecholamines in brain adrenals and urine

Adrenaline isolated in 1901 by TAKAMINE is the main active principle in the adrenal medulla of mammals It was synthesized in 1904 by STOLZ as was also noradrenaline Adrenaline was long thought to be the main sympathetic transmitter as suggested by ELLIOTT (1905)

The physiological importance of noradrenaline was clarified much later (EULER 1946) when it was shown that the active sympathomimetic principle consists chiefly of noradrenaline. Up to that time all measurements of catecholamines discharged from sympathetic nerve terminals had been measured as adrenaline generally by bioassay methods

In 1947 HOLTZ CREDNER and KRONEBERG discovered the presence of noradrenaline in normal urine The main source of the urinary noradrenaline secreted under resting conditions is probably the adrenergic nerves while the small amounts of adrenaline originate from the adrenal glands (EULER 1956 p 287) A massive discharge mainly of adrenaline may occur in emergency states

EULER (1946) first demonstrated the presence of small amounts of catecholamines mainly noradrenaline in the brain This observation was extended by VOGT (1954) who found that the brain catecholamines were unevenly distributed The highest concentrations were found in the hypothalamus and somewhat less in the grey stratum around the aqueduct The concentration of noradrenaline in the grey matter of the telencephalon was only about 5 per cent of that of the hypothalamus, while the medullated fibres contained still less The very high concentrations of noradrenaline in certain parts of the brain suggested a possible transmitter function of noradrenaline in the brain tissue in addition to its role as a transmitter at vasomotor endings Recently the catecholamine stores of hypothalamic nuclei have been visualized by histochemical methods through the work of CARLSSON, FALCK and HILLARP (1962)

The presence of dopamine (3 hydroxytyramine) in the mammalian brain was suggested by MONTAGU (1957) and proved through independent work by EULER (1958) and CARLSSON *et al* (1958) BERTLER and

ROSENGREN (1959) investigated the distribution of dopamine in the brain and found high concentrations in the central ganglia of the telencephalon, notably the caudate nucleus The substance was tentatively correlated with locomotor functions of the extrapyramidal system It is generally agreed that the dopamine of the brain serves also as a precursor of noradrenaline

The relative contribution of adrenaline to the mammalian brain "sympathin" has been a matter of differences in opinion HOLTZ (1950) and VOGT (1954), using bioassay methods, obtained relative values for adrenaline between 8 and 25 per cent (of the sum of noradrenaline and adrenaline) MONTAGU (1956 a) found 34 per cent adrenaline in the rat brain, while BERTLER and ROSENGREN (1959) were unable to verify these high adrenaline levels in mammalian brains and found appreciable amounts only in the brains of amphibia

Occurrence of 5 hydroxytryptamine in brain and 5 hydroxyindoleacetic acid in urine

The presence of 5 hydroxytryptamine (5 HT, serotonin, enteramine) in the brain was first reported by TWAROG and PAGE (1953) AMIN, CRAWFORD and GADDUM (1954) reported a distribution of this substance in the brain very similar to that of noradrenaline, the largest amounts being stored in the hypothalamus TITUS and UDENFRIEND (1954) and ERSPAMER (1954 a) have investigated the fate of 5 hydroxytryptamine in the organism They found that the most important metabolic route was the oxidative deamination by *monoamine* oxidase, with 5 hydroxyindoleacetic acid (5 HIAA) as the metabolic end product, appearing in the urine Most of the 5 hydroxytryptamine in the body is stored in the intestinal mucosa and the blood platelets (FELDBERG and TOH 1953, GADDUM 1954), from which the bulk of the urinary 5 HIAA is derived

The brain stores of 5 hydroxytryptamine although quantitatively insignificant, have nevertheless received much attention It was speculated (GADDUM 1954, WOOLLEY and SHAW 1954), that this substance would have a role in brain function possibly as a neurohumoral agent (WELSH 1954, BRODIE, PLETSCHER and SHORE 1955) Following the release of 5 hydroxytryptamine, it is attacked by the enzyme monoamine oxidase, which is present in the brain and 5 HIAA is formed Since this acid passes slowly through the blood brain barrier the 5 HIAA of the brain is a measure of the local release of 5 hydroxytryptamine (ROOS and WERDINIUS 1962)

In 1952 ZELLER and BARSKY, studying the monoamine oxidase inhibiting properties of some hydrazine derivatives suggested that the effect of these drugs on the monoamines might have some relation to the sympathetic and general mental stimulation observed A few years later, when the monoamines of the brain had been mapped out this suggestion gained new interest

During the last few years several compounds have been developed which act upon the synthesis storage and break down of the brain monoamines By interference with any one of these mechanisms it has been possible to produce states of excitement in various laboratory animals Corresponding signs have also been found in man (DEGKWITZ *et al* 1960)

Synthesis According to a now widely accepted theory first suggested by BLASCHKO 1939 the biosynthesis of the catecholamines occurs according to the following scheme

tyrosin → DOPA → dopamine → noradrenaline → adrenaline

The enzyme DOPA decarboxylase is present in the brain (HOLTZ and WESTERMANN 1956) where it converts DOPA (3 4-dihydroxyphenyl alanine) to dopamine Probably the same enzyme (ROSENGREN 1960 a) attacks also 5 hydroxytryptophan (5HTP) the precursor of 5 hydroxy tryptamine The decarboxylation is a rapid process (UDENFRIEND 1959) which takes place as soon as the amino acids are present in the brain The administration of 5 HTP or DOPA thus affords a means of increasing the rate of synthesis of 5 hydroxytryptamine or the catecholamines in the brain (UDENFRIEND BOGDANSKI and WEISSBACH 1956, CARLSSON *et al* 1958) The amines themselves do not readily pass the blood brain barrier (WEIL MALHERBE AXELROD and TOMCHICK 1959 WILSON and BRODIE 1961)

When 5 HTP is administered to rats there is an increase in the 5 hydroxytryptamine content of the brain and at the same time a state of excitation is noted (UDENFRIEND WEISSBACH and BOGDANSKI 1957) Following administration of DOPA a corresponding increase of brain dopamine is seen together with central excitation (CARLSSON *et al* 1958, BERTLER 1960)

Attempts to decrease the synthesis of brain monoamines by inhibition of the decarboxylating enzyme have not been successful *in vivo* (BRODIE *et al* 1962) Some of the potent *in vitro* inhibitors have been shown to act by other mechanisms as releasers of central catecholamines (UDENFRIEND, CONNAMACHER and HESS 1961)

Storage The monoamines of the brain are stored in an inactive form Following liberation they are attacked by the catabolizing enzymes Four groups of agents which act upon the size of these stores are exemplified

1) A release can be produced by *intense stimulation through nervous mechanisms* This seems to be the mechanism of action of a series of different compounds, like insulin, nicotine and *morphine* (VOGT 1954) *The* common denominator of these compounds is the stimulation of sympathetic nerves

The stimulation effects a reduction of the catecholamine level of the brain, while 5 hydroxytryptamine remains unaffected (PAASONEN and VOGT 1956) The catecholamine level is rapidly restored when the stimulation is ended

2) Release can be effected by interfering with the amine binding capacity of the tissues An example of an agent which operates by this mechanism is reserpine In contrast to the agents in group 1 (above) reserpine produces a *long-lasting* depletion of the brain content of both 5 hydroxytryptamine (PLETSCHER, SHORE and BRODIE 1956 b, PAASONEN and VOGT 1956), *noradrenaline* (HOLZBAUER and VOGT 1956) and dopamine (CARLSSON *et al* 1958, WEIL MALHERBE and BONE 1958) It is well known that this *depletion is accompanied by marked sedation and loss of spontaneous* activity

The activity can be *restored* by DOPA *but not by* 5 hydroxytryptophan (CARLSSON LINDQVIST and MAGNUSSON 1957), thus suggesting that reserpine may act by producing a lack of central transmitter There are also, however, other theories regarding these mechanisms, which involve 5 hydroxytryptamine (BRODIE *et al* 1957, GESSA *et al* 1962) or its deaminated end product (HOLTZ *et al* 1957) as the centrally acting sedative substance

3) A third way of releasing the brain stores of monoamines is illustrated *by a methyl* DOPA *which is known* to have mildly sedative and blood pressure reducing properties This substance is metabolized *in vivo* to *a* methylated catecholamines leading eventually to a long lasting displacement of noradrenaline by *a* methyl noradrenaline a substance which *is not* attacked by monoamine oxidase (CARLSSON and LINDQVIST 1962) Dopamine and 5 hydroxytryptamine are moderately reduced only for a few hours by the *a* methylated catechol *analogues* (SOURKES *et al* 1961 PLETSCHER and GEY 1962)

4) The monoamine oxidase inhibitors have been shown to act not only

the release of the amines from their binding sites in the tissues (AXELROD, HERTTING and PATRICK 1961)

Catabolism The major metabolic pathway of the monoamines in the mammalian brain seems to be the oxidative deamination by monoamine oxidase (SPECTOR *et al* 1960, ROSENGREN 1960 b GEY and PLETSCHER 1961) Catecholamines released into the peripheral blood or administered exogenously are inactivated primarily by the catechol O methyltransferase (COMT) of the liver and kidney (AXELROD 1959) This enzyme was shown to occur also in small concentrations in the brain (AXELROD 1958) In a study by CARLSSON and HILLARP (1962) the administration of DOPA to rabbits caused initially an increase in the brain of the monoamine oxidase product 3 4 dihydroxyphenylacetic acid (DOPAC), which was transformed secondarily to the methoxylated COMT product, homovanillic acid This finding supports the view that the catecholamines of the brain are primarily attacked by monoamine oxidase

Administration of monoamine oxidase inhibitors results in an increased brain level of 5 hydroxytryptamine (for which monoamine oxidase is probably the only metabolic pathway) The noradrenaline of the brain increases in some species (rats, dogs) but remains unaffected in others (cats for instance) following administration of monoamine oxidase inhibitors When the noradrenaline level is high there is generally a more or less marked excitement (BRODIE SPECTOR and SHORE 1959 b) The excitement is increased by administration of DOPA (CARLSSON *et al* 1957), 5 HTP (UDENFRIEND *et al* 1957) and by reserpine (SHORE and BRODIE 1957) or the *a* methylated catechol analogues (COSTA *et al* 1962) All these substances produce excitement probably by increasing the amount of free amines at synaptic sites Whether dopamine is the most important of the catecholamines in this respect cannot be regarded as settled BERTLER (1960) demonstrated a close correlation in time between the dopamine content of the brain and the signs of excitement after administration of DOPA in rabbits

For 5 hydroxytryptamine the relation to states of excitement is more obscure A central tranquilizing action has been ascribed to it (SULSER and BRODIE 1960 GESSA *et al* 1962)

Effects of morphine on catecholamines and 5 hydroxytryptamine

ELLIOTT (1912) reported an adrenaline-depleting action of morphine in the adrenal glands of cats and dogs It was noticed that the effect could be prevented by sectioning the splanchnic nerve before the administration of morphine and thus that it was mediated through nervous stimulation

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1) A release can be produced by intense stimulation through nervous mechanisms This seems to be the mechanism of action of a series of different compounds, like insulin, nicotine and morphine (VOGT 1954) The common denominator of these compounds is the stimulation of sympathetic nerves

The stimulation effects a reduction of the catecholamine level of the brain, while 5 hydroxytryptamine remains unaffected (PAASONEN and VOGT 1956) The catecholamine level is rapidly restored when the stimulation is ended

2) Release can be effected by interfering with the amine binding capacity of the tissues An example of an agent which operates by this mechanism is reserpine In contrast to the agents in group 1 (above) reserpine produces a long lasting depletion of the brain content of both 5 hydroxytryptamine (PLETSCHER, SHORE and BRODIE 1956 b, PAASONEN and VOGT 1956), noradrenaline (HOLZBAUER and VOGT 1956) and dopamine (CARLSSON *et al* 1958, WEIL-MALHERBE and BONE 1958) *It is well known that this* depletion is accompanied by marked sedation and loss of spontaneous activity

The activity can be restored by DOPA but not by 5 hydroxytryptophan (CARLSSON, LINDQVIST and MAGNUSSON 1957), thus suggesting that reserpine may act by producing a lack of central transmitter There are also, however, other theories regarding these mechanisms, which involve 5 hydroxytryptamine (BRODIE *et al* 1957, GESSA *et al* 1962) or its deaminated end product (HOLTZ *et al* 1957) as the centrally acting sedative substance

3) A third way of releasing the brain stores of monoamines is illustrated by *a* methyl DOPA which is known to have mildly sedative and blood pressure reducing properties This substance is metabolized *in vivo* to *a* methylated catecholamines leading eventually to a long lasting displacement of noradrenaline by *a* methyl noradrenaline, a substance which is not attacked by monoamine oxidase (CARLSSON and LINDQVIST 1962) Dopamine and 5 hydroxytryptamine are *moderately reduced only for a* few hours by the *a* methylated catechol analogues (SOURKES *et al* 1961 PLETSCHER *and* GEY 1962)

4) The monoamine oxidase inhibitors have been shown to act not only on the metabolic break down of the catecholamines, but also by preventing

CHAPTER II

GENERAL METHODS

1 Experimental procedures

Experimental animals

Dogs rats and cats were used in the present investigation The animals were fed a balanced commercial brand of laboratory food, and were allowed food and tap water *ad libitum*

In the experiments to be described 45 mongrel *dogs* of either sex were used The animals ranged in weight from 4 to 15 kg Approximately the same number of males and females was placed in each experimental group The dogs were sacrificed by a gun shot through the heart

A total of 330 male albino *rats* of the Sprague Dawley strain (Anticimex, Stockholm) were employed When possible adult rats weighing 250—300 g were used The weights of the animals in each group did not vary more than ± 10 g at the beginning of an experiment The rats were sacrificed by decapitation

Fifteen *cats* of either sex weighing 2 5—4 0 kg were used in acute and short term morphine experiments The cats were sacrificed by decapitation following chloroform anaesthesia

Administration of morphine and nalorphine

A 4 per cent solution of morphine hydrochloride rendered isotonic with 0 3 per cent of sodium chloride was used For stabilization of the morphine hydrochloride 0 1 per cent of 1 N hydrochloric acid was added to obtain a final pH around 4 This solution is referred to as *morphine* throughout this paper

Nalorphine was given as a 1 per cent solution of nalorphine hydrochloride (Nalorphin Leo Ltd)

Dogs and cats were given subcutaneous injections while rats received intraperitoneal injections of morphine and nalorphine

Rats and cats were used in acute morphine experiments The injections were given at 9 a m and the animals were sacrificed four hours later In a few experiments rats and cats were treated daily with morphine for only a few days The rats were injected twice daily at 9 a m and 5 p m and

The observation that morphine reduces the adrenaline content of the adrenals and/or elevates the catecholamine content of the adrenal vein blood, has been confirmed in cats (STEWART and ROGOFF 1922, EMMELIN and STROMBLAD 1951, VOGT 1954), rabbits (ABE 1929, TADA 1932), rats (OUTSCHOORN 1952) and dogs (SATO and OHMI 1933, WADA *et al* 1938, SIBUTA, ENDO and NAGAKURA 1949)

An increase in the adrenaline concentration of peripheral blood after morphine injection was reported in rabbits (HAYAMA 1932) and dogs (RICHARDSON, WOODS and RICHARDSON 1958) The urinary output of catecholamines increased after morphine injection in rats (CRAWFORD and LAW 1958)

ABE (1929) found an increased amount of adrenaline in the adrenal glands of morphine tolerant rabbits and a disappearance of the adrenaline depleting effect after long term morphine treatment TACHIKAWA (1932) noticed that in the blood of chronically morphine treated dogs "there is a greater amount of adrenaline in the non morphine period WADA *et al* (1938) reported that the morphine induced output of adrenaline in the adrenal vein blood of dogs was diminished as a result of morphine tolerance

VOGT (1954) reported that, in addition to the catecholamine depletion of the adrenal glands, there was a concomitant reduction of the midbrain and hypothalamic stores of sympathin in cats given 30—60 mg/kg of morphine In dogs there was only a slight corresponding effect QUINN, BRODIE and SHORE (1958) confirmed this observation in cat brains but found no effect of morphine on the catecholamines of rabbit brains

5-Hydroxytryptamine was not changed in the rabbit brain after single morphine doses (BRODIE, SHORE and PLETSCHER 1956) Long term administration of morphine in rats did not influence the brain 5 hydroxytryptamine (COCHIN and AXELROD 1959)

No reports dealing with the effects of long term morphine administration or withdrawal on the brain catecholamines seem to have appeared before 1959, when my first paper on this subject was published Some recent reports are discussed later in the present work — No earlier studies except my own 1961 1962 a, b) of the urinary output of catecholamines and 5 HIAA in morphine tolerance and withdrawal are known to the author

phine Dogs were given two consecutive injections of 5 + 5 mg/kg at 9 a m and 11 a m while rats received one injection of 10 mg/kg The animals were sacrificed four hours after the (first) nalorphine injection

c The animals were withdrawn from the regular morphine injections dogs for 72 hours and rats for 48 hours before sacrifice

One group of *control dogs* was untreated Another control group received two consecutive injections of nalorphine 5 + 5 mg/kg at two hours intervals and was sacrificed four hours after the first injection The *control rats* were given saline in amounts corresponding to the morphine treated animals One group of control rats received in addition a single injection of 10 mg/kg of nalorphine and was sacrificed four hours later

When urine was collected from four morphine tolerant dogs the animals were stabilized on single daily morphine injections 90 mg/kg given at 9 a m This morphine dose was substituted by one injection of nalorphine 10 mg/kg whereafter the morphine administration was resumed

Collection of urine

When dogs and rats were kept in metabolic cages special precautions had to be taken to avoid fragmentation of food into small particles which might absorb considerable amounts of urine During these periods the dogs were fed only meat while the rats diet was ground and thoroughly mixed with water

Young *dogs* weighing 3—5 kg were housed in individual metabolic cages The floor consisted of cylindrical plexiglass rods 10 mm in diameter inserted at 5 mm distances from each other The urine was collected in glass flasks through plexiglass funnels in which a plug of pyrex wool was placed to filter out fecal solids

Fifty ml of 1 N sulphuric acid were usually added to the bottles every 24 hours to produce an approximate pH of 3 The cages were cleaned daily for ½ hour during which time the animals were allowed to move freely For convenience the 23½ hours urine was calculated as 24 hours urine

Since no special measures were observed to avoid leakage of drinking water directly into the funnels the values for the urine volumes may have been too high However, together with the salivation of the dogs the additional volume probably amounted only to a few per cent When vomiting occurred as in one dog during nalorphine induced abstinence this animal was excluded when the mean urine volumes were calculated — Naturally the calculation of the urinary amines was uninfluenced by these sources of error

the cats once daily These animals were sacrificed four hours after the last injection, given at 9 a m

Dogs and rats were given chronic morphine treatment The initial morphine dose in the *dogs* was 3 mg/kg given once daily at 9 a m This dose was increased at intervals of 7 to 10 days until 40 mg/kg were reached after 6 to 8 weeks After this the dose was divided and given at 9 a m and 5 p m The morning dose was half the evening dose During the first week of split doses, 20 mg/kg were given in the morning and 40 mg/kg in the afternoon, next week the doses were 30 and 60 mg/kg, etc The final dose was 90 to 120 mg/kg/day and the whole series of injections lasted for 70 to 90 days In a few instances some dogs were readdicted after withdrawal for three weeks The second cycle of addiction was shortened to about four weeks

This schedule of administration is referred to as *chronic* morphine treatment throughout this paper Dogs given chronic morphine treatment are referred to as *morphine tolerant*

The *rats* were injected for three weeks according to two different dosage regimens

1 *Standard regimen (20—180 mg/kg twice daily)*

Injections were given twice daily at 9 a m and 5 p m starting with 20 mg/kg/injection The dose was tripled every seventh day and the dose levels of the three weeks were thus 20, 60 and 180 mg/kg/injection

2 *Intense regimen (20—300 mg/kg at 4 hours intervals)*

During the first week injections were given twice daily at 9 a m and 5 p m of 20 mg/kg/injection From the eighth day five daily injections were given at 7 a m, 11 a m, 3 p m, 7 p m and 11 p m The dose was increased by 20 mg/kg/injection every day (20, 40, 60, 80 mg/kg/injection) After fourteen days the rats were given six daily injections every fourth hour around the clock until, on the twentyfirst day they received 300 mg/kg/injection, corresponding to a daily dose of 1 800 mg/kg

These two schedules of morphine administration are referred to as *chronic* treatment and rats so treated as *morphine tolerant* rats

When dogs and rats had been rendered morphine tolerant, the following treatments were employed

a The regular morphine injection at 9 a m was substituted by one injection of saline Dogs were given an additional saline injection at 11 a m and the animals were sacrificed four hours after the first saline injection

b The regular morphine injection at 9 a m was substituted by nalor-

phine Dogs were given two consecutive injections of 5 + 5 mg/kg at 9 a m and 11 a m , while rats received one injection of 10 mg/kg The animals were sacrificed four hours after the (first) nalorphine injection

c The animals were withdrawn from the regular morphine injections dogs for 72 hours and rats for 48 hours before sacrifice

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Five rats were placed in each metabolic community cage. There were no metal parts in the collecting system. The floor, 50 cm in diameter, was mad of perforated plexiglass and placed on a glass funnel with arrangements for separation of fecal solids Ten ml of 1 N sulphuric acid were added, as a preservative, to the polythene collecting bottles

2 Estimation of catecholamines

Noradrenaline and adrenaline were estimated in the brains and adrenals of dogs, cats and rats and the urine of dogs and rats Dopamine was estimated in the brains of dogs

Choice of method

For determination of catecholamines in the brains of dogs and cats a modification of the method of EULER and LISHAJKO (1961) was used A preliminary study (*cf* below) showed that the results with this convenient method were almost identical with those obtained by the somewhat more time consuming method of BERTLER, CARLSSON and ROSENGREN (1958)

Estimation of catecholamines in the rat brain was made by the method of BERTLER *et al* (1958) for the following reasons — The experiments with rats included administration to the animals of *a* methyldihydroxy phenylalanine (*a* methyl DOPA) a substance which is converted in the brain to *a* methylated catecholamines (CARLSSON and LINDQVIST 1962) Alpha methyl dopamine, when treated according to the trihydroxyindole (THI) method, gives a strongly fluorescent product, which would invalidate the estimation of noradrenaline Therefore it was thought advisable to exclude interference from *a*-methyl dopamine by the chromatographic separation on Dowex 50 The fluorophore of *a* methyl noradrenaline, on the other hand which is not separated from noradrenaline by this method had a weak fluorescence, less than 5 per cent of that of noradrenaline with the filter sets used in the present investigation This is in accordance with earlier reports (LUND 1949, CARLSSON and LINDQVIST 1962)

Dopamine was estimated in the telencephalon of dogs according to CARLSSON and WALDECK (1958)

The extracts of adrenals were diluted with sodium acetate buffer solution and the determination of adrenaline and noradrenaline was then performed as for brain and urinary eluates

Free urinary adrenaline and noradrenaline were determined by the method of EULER and LISHAJKO (1961)

A Extraction

Extraction of brain tissue was performed with 0 4 N perchloric acid Only one extraction was made and the final catecholamine values were calculated according to BERTLER *et al* (1958), presuming a water content of the tissue of 75 per cent This implies the following correction factor for the catecholamine values found

(Extraction vol + 75 % of wet tissue wt)/(vol taken for assay)

The *dog brain* was dissected out immediately after sacrifice stripped of the meninges and divided into three parts 1) telencephalon 2) cerebellum and 3) brain stem (medulla oblongata pons midbrain and diencephalon but excluding the basal ganglia belonging to the telencephalon) Each part was weighed and homogenized (Ultra Turrax homogenizer) in six volumes of cold 0 4 N perchloric acid The extraction was allowed to proceed for about 30 min at +4° C after homogenization After centrifugation the supernatant was passed through a filter paper (Munktell density grade 00) Generally 40 ml of the extracts of telencephalon and cerebellum and 15 ml of the brain stem extracts were taken for purification and assay

The whole *rat brains* were dissected out and put in 0 4 N perchloric acid Two brains were pooled for each determination homogenized and extracted in 20 ml (six volumes) of cold acid The extracts were treated as described above for dog brains All extracts were stored at −30° C for not more than one week The whole extract was taken for assay

The *cat brain* was removed and the brain stem (here defined as medulla oblongata pons midbrain and diencephalon) was cut out It was extracted with six volumes of 0 4 N perchloric acid and treated as described for the dog brains

The *adrenals* of dogs rats and cats were dissected out 15 to 30 min after death The organs were carefully freed from adherent tissue weighed on a torsion balance and ground in a mortar with quartz sand together with 5 per cent trichloroacetic acid Adrenals of dogs (one pair) and cats (one pair) were extracted in 50 ml For the rats adrenals (two pairs) 10 ml were used After filtration the extracts were generally stored at −30° C for 1 to 7 days before further dilution and fluorimetric estimation.

Stability of noradrenaline in perchloric acid extracts

The pH of the perchloric acid brain extracts was about 0 5 The studies

of WEST (1952) indicate, however, an optimal stability for catecholamines at pH 3.5 to 3.9. In order to study the stability of noradrenaline in the perchloric acid extracts stored at −30° C, the following experiment was undertaken.

The brain stems of three dogs, with a total tissue weight of 25 g were extracted with 150 ml of 0.4 N perchloric acid. The pooled extract was divided in ten parts of 15 ml each. Two samples were determined on the day of extraction to obtain reference values. Four samples were adjusted to pH 3.8 with 2 N potassium hydroxide, and together with the four extracts at pH 0.5 they were stored at −30° C. At intervals of one month two samples were taken out, one at pH 3.8 and one at pH 0.5 and determined as above.

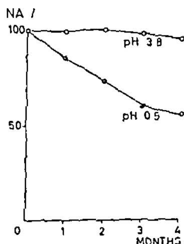


Fig. 1. Stability of noradrenaline (NA) in 0.4 N perchloric acid extract of dog brains (pH 0.5). Part of the pooled extract was adjusted to pH 3.8 before storage at −30° C.

The *results* are given in Fig. 1 from which it is evident that 3.8 is a more favourable pH for storage. At pH 0.5 the noradrenaline content was reduced to about 50 per cent in four months. The gradual reduction of noradrenaline was obviously not prevented by storage at −30° C.

In accordance with BERTLER *et al.* (1958), *it was found that the samples* could be stored for a week without appreciable loss. If the time for storage was extended beyond a week, the samples were adjusted to pH 3.8.

B. Purification

As mentioned previously, the brain extracts were purified according to two different methods. These will be briefly described below.

Method of BERTLER, CARLSSON *and* ROSENGREN *(1958)*

A column 4 2 mm in diameter containing 300 mg of the strongly acid cation exchange resin Dowex 50 W X4 200—400 mesh Na form was used The resin had been washed with 5 N hydrochloric acid glass distilled water and 1 N sodium hydroxide Fourty ml of 2 N HCl redistilled water and 1 N sodium acetate buffer pH 6 0 were passed through the column prior to analysis

The perchloric acid extracts were thawed and adjusted to pH 4 with 2 N potassium hydroxide A pH meter (Metrohm) and a magnetic stirrer were used in all titrations The precipitate of potassium perchlorate was spun down at 0° C and the extract was passed through the columns

After two washings with glass distilled water elution was performed with 1 N hydrochloric acid in two separate portions the first 9 ml of the eluate contained noradrenaline and adrenaline The second portion of the eluate (10 ml) contained approximately 90 per cent of the dopamine content of the sample

The same column could be used again after rinsing with 2 N hydrochloric acid and regeneration as described in the original method

Method of EULER *and* LISHAJKO *(1959 and 1961)*

The perchloric acid extracts were thawed adjusted to pH 4 with 2 N potassium hydroxide and the perchlorate was spun down The pH of the supernatant was adjusted to 8 4 with 0 1 N sodium hydroxide and the extract was passed through a column 0 9 cm in diameter containing 1 g of aluminium oxide (British Drug Houses) previously washed with glass-distilled water to let fine particles pass through the glass filter After adsorption the column was carefully washed with glass distilled water and elution was performed with 0 25 N acetic acid

When the extracts were clear and colorless (as for instance dog brain stem and cerebellum extracts) the potassium hydroxide titration and centrifugation steps could be omitted and pH adjusted to 8 4 directly by 1 N sodium hydroxide

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Adrenal extracts could be assayed for catecholamines after dilution without purification The extract was thawed and thoroughly stirred in order to avoid the separation brought about by freezing (the cryoscopic effect RANGAIPA 1961) For *dogs'* adrenals 0 1 ml of the extract was generally diluted in 9 9 ml 0 15 N sodium acetate buffer at pH 6 5 For the *rats'* and *cats* adrenals 0 5 ml of extracts was diluted in 9.5 ml 1 N sodium acetate buffer at pH 6 8 After dilution the final pH before assay was 6 3 in all extracts

The *urine* of dogs and rats was filtered whereafter 25 ml or less were treated according to the method of EULER and LISHAJKO (1959), including addition to the urine of the disodium salt of ethylenediaminetetraacetic acid (EDTA)

of *West* (1952) indicate, however, an optimal stability for catecholamines at pH 3 5 to 3 9 In order to study the stability of noradrenaline in the perchloric acid extracts stored at $-30°$ C, the following experiment was undertaken

The brain stems of three dogs, with a total tissue weight of 25 g were extracted with 150 ml of 0 4 N perchloric acid The pooled extract was divided in ten parts of 15 ml each Two samples were determined on the day of extraction to obtain reference values Four samples were adjusted to pH 3 8 with 2 N potassium hydroxide, and together with the four extracts at pH 0 5 they were stored at $-30°$ C At intervals of one month two samples were taken out, one at pH 3 8 and one at pH 0 5 and determined as above

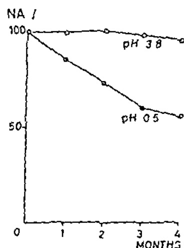


Fig 1 Stability of noradrenaline (NA) in 0 4 N perchloric acid extract of dog brains (pH 0 5) Part of the pooled extract was adjusted to pH 3 8 before storage at -30 C

The *results* are given in Fig 1 from which it is evident that 3 8 is a more favourable pH for storage At pH 0 5 the noradrenaline content was reduced to about 50 per cent in four months The gradual reduction of noradrenaline was obviously not prevented by storage at $-30°$ C

In accordance with *Bertler et al* (1958) it was found that the samples could be stored for a week without appreciable loss If the time for storage was extended beyond a week the samples were adjusted to pH 3 8

B Purification

As mentioned previously, the brain extracts were purified according to two different methods These will be briefly described below

Table II Comparison between purification on Alumina and Dowex 50 of a solution of noradrenaline (NA) 2 μg/10 ml in perchloric acid

	Alumina NA μg/10 ml	Dowex 50 NA μg/10 ml
	1 68	1 70
	1 69	1 74
	1 73	1 76
	1 78	1 76
	1 78	1 82
Means	1 73	1 76
Recovery	87 %	88 %

Standard solutions To a solution of 0 4 N perchloric acid noradrenaline hydrochloride was added 2 μg/10 ml and potassium chloride *corresponding* to the approximate ion concentration of a brain extract Five samples of 15 ml of this simulated brain extract were adjusted to pH 4 with a measured volume of 2 N KOH centrifuged and a 10 ml aliquot of the extract was passed through a column of Dowex 50 (This procedure eliminates the losses normally included in the centrifugation step) Five *other samples* were adjusted to pH 8 4 by 1 N sodium hydroxide and passed through columns of alumina The eluates were assayed fluorimetrically as described below

The values expressed as μg/10 ml of the extract show that both methods gave almost identical results (Table II)

Table III Comparison between purification on Alumina and Dowex 50 of noradrenaline (NA) in brain stem extracts of dogs The pooled extract of untreated dogs divided into 10 equal samples

	Alumina NA μg/g	Dowex 50 NA μg/g
	0 35	0 32
	0.35	0 37
	0 36	0 33
	0 36	0 34
	0 35	0 32
Means	0 35	0 33

Addition of EDTA

When the disodium salt of ethylenediaminetetraacetic acid (EDTA, Titriplex, Merck) was added to the brain extracts, in amounts corresponding to those recommended for the same volumes of urine (about 140 mg/g of wet tissue), the noradrenaline values were considerably depressed In order to secure the relationship between the lowered values and the addition of EDTA the following study was undertaken

The pooled extract of ten rat brains in 100 ml 0 4 N perchloric acid was divided in five equal samples to which various additions of EDTA were made The samples were purified on alumina and assayed according to EULER and LISHAJKO (1961) It was found that even small amounts of EDTA lowered the noradrenaline values (Table I) Addition of EDTA

Table I Influence of added amounts of the disodium salt of ethylene diaminetetraacetic acid (EDTA) on the noradrenaline (NA) content in rat brains Extracts of 10 rat brains pooled and divided in 5 equal samples

EDTA mg/g	NA %
0	100
5	70
10	68
15	50
50	50

was for this reason omitted for brain extracts and was found unnecessary when the extraction volume was sufficiently large to prevent precipitation (of phosphates and other salts) at pH 8

When treating the urine samples, EDTA could not be dispensed with, unless the urine was either diluted to avoid precipitating salts or centrifuged in order to remove the precipitate at pH 8 4 These steps lowered the recovery yields and were abandoned

Comparison between the methods of EULER *and* LISHAJKO *(1961) and* BERTLER, CARLSSON *and* ROSENGREN *(1958)*

As two different methods of purification of the brain extracts were used, the following studies were undertaken to evaluate the accuracy and specificity of these methods

levels obtained after purification on Dowex 50. This is in accordance with earlier results (GUNNE 1961) when morphine was added to urine and the purification was made on alumina.

Stability of eluates. Since the pH of the hydrochloric acid eluates of the method of BERTLER *et al.* (1958) was found to approximate 0.5 the samples were probably not stable for any length of time (*cf.* Fig. 1). For this reason the final assay was made without delay on the day of elution.

The acetic acid eluates of the method of EULER and LISHAJKO (1961) had a pH around 3.5 and could be stored in the cold for months without appreciable loss.

These studies indicate that for the purpose of the present investigation, the two methods of purification gave results which were comparable with regard to accuracy and specificity.

C. Assay

The assay of adrenaline and noradrenaline in the final eluates was performed by fluorimetric technique using the trihydroxyindole (THI) method (EHRLÉN 1948, LUND 1949, 1950, EULER and FLODING 1955). This includes oxidation of the catecholamines and rearrangement of the oxidized products in alkaline solution to the corresponding strongly fluorescent lutines using ascorbic acid as a stabilizing agent. Blanks were prepared either by omitting the ascorbic acid until the fluorescence had disappeared (faded blank) or by omitting the oxidation step (non-oxidized blank). Though both methods for preparing the blank gave comparable results the faded blanks were found to be more stable and were chosen for this reason for all eluates. The non-oxidized blanks were used only for analysis of adrenal extracts which presumably contain very little interfering material.

The final determination of noradrenaline and adrenaline was made on a Coleman fluorimeter type 12 C using two sets of monochromatic filters as described by COHEN and GOLDENBERG (1957). The filter set *a* consisted of a primary monochromatic filter with a peak transmission at 395 nm (the filter was a combination of Schott UG 3, BG 14, GG 13) and as a secondary filter Ilford Bright 623 with peak transmission at 490 nm. The filter set *b* consisted of a primary monochromatic filter with peak transmission at 436 nm (the filter was a combination of Schott BG 12, GG 15) and as secondary filter Corning 3486 with peak transmission 540 nm

Table IV Comparison between purification on Alumina and Dowex 50 of noradrenaline (NA) in brain stem extracts of dogs The dogs had been given chronic morphine (Mo) treatment, final dose 100 mg/kg Three dogs received 5+5 mg/kg of nalorphine before sacrifice

Treatment	Alumina NA μg/g	Dowex NA μg/g	Difference
Chronic Mo	0 34	0 35	-0 01
„	0 30	0 30	0
„	0 32	0 30	+0 02
Chronic Mo+Nalorphine	0 24	0 21	+0 03
„	0 12	0 13	-0 01
„	0 25	0 22	+0 03

Brain extracts of untreated dogs Two hundred ml of pooled extract from brain stems of untreated dogs were divided in ten samples of 20 ml each Five of the extracts were treated by the method of BERTLER *et al* (1958) and five according to EULER and LISHAJKO (1961) The results with both methods were practically the same (Table III)

Brain extracts of chronically morphine treated dogs The brain stem of three chronically morphine treated dogs were extracted with 0 4 N perchloric acid From each extract two 15 ml samples were taken for purification and determination by each method The same experiment was repeated in three other chronically morphine treated dogs who had received in addition two injections of nalorphine, 5 mg/kg, four and two hours before examination In spite of the different treatments there was still a satisfactory agreement in the results obtained by both methods (Table IV)

Addition of morphine to brain extract The pooled extract of eight rat brains was divided in four equal parts To two of these, morphine hydrochloride was added in amounts corresponding to 40 μg/g wet tissue and the extracts were treated by the method of BERTLER *et al* (1958) Other workers (JOHANNESON 1962 and MILTHERS 1962) have shown that higher doses of morphine than those used in the present investigation produce brain levels approximating this concentration

The addition of morphine HCl did not influence the catecholamine

evels obtained after purification on Dowex 50 This is in accordance with earlier results (GUNNE 1961), when morphine was added to urine and the purification was made on alumina

Stability of eluates Since the pH of the hydrochloric acid eluates of the method of BERTLER *et al* (1958) was found to approximate 0 5 the samples were probably not stable for any length of time (*cf* Fig 1) For this reason the final assay was made without delay on the day of elution

The acetic acid eluates of the method of EULER and LISHAJKO (1961) had a pH around 3 5 and could be stored in the cold for months without appreciable loss

These studies indicate that, for the purpose of the present investigation the two methods of purification gave results which were comparable with regard to accuracy and specificity

C Assay

The assay of adrenaline and noradrenaline in the final eluates was performed by fluorimetric technique, using the trihydroxyindole (THI) method (EHRLEN 1948 LUND 1949 1950 EULER and FLODING 1955) This includes oxidation of the catecholamines and rearrangement of the oxidized products in alkaline solution to the corresponding strongly fluorescent lutines using ascorbic acid as a stabilizing agent. Blanks were prepared either by omitting the ascorbic acid until the fluorescence had disappeared (faded blank) or by omitting the oxidation step (non-oxidized blank) Though both methods for preparing the blank gave comparable results the faded blanks were found to be more stable and were chosen for this reason for all eluates The non-oxidized blanks were used only for analysis of adrenal extracts which presumably contain very little interfering material

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A plexi-glass rod was used as standard which was repeatedly calibrated

against solutions containing the fluorophores of noradrenaline and adrenaline A recalibration was required every 2 to 3 weeks

Using these filter sets the fluorescence of noradrenaline is approximately equal with *a* and *b* filters, while the fluorescence of adrenaline is about three times higher with *b* filters compared with *a* filters

A slow gradual change was noticed in the sensitivity of the fluorimeter probably as a result of changes in the transmission qualities of the filters It was found that this gradual change followed a pattern which was to a certain extent predictable It implied an inherent tendency of the instrument to obtain an increased sensitivity to adrenaline If the plexiglass standard was not checked against catecholamine standards for some interval the values were biassed in the direction of too high adrenaline figures while the noradrenaline values were still approximately correct

Dopamine was estimated according to CARLSSON and WALDECK (1958) The fluorescence was read in a spectrophotofluorimeter (Aminco Bowman) at the peak fluorescence wavelengths and compared with known standard solutions

Procedure

The hydrochloric acid eluates were titrated to pH 6 0—6 5 with 2 N potassium hydroxide, after addition of 0 1 ml 5 N acetic acid to obtain an increased buffer capacity (Titration with potassium carbonate, as recommended by BERTLER *et al* (1958), was not used since it was found difficult to obtain a stable pH in spite of rapid stirring with a magnetic stirrer) The acetic acid eluates were titrated with 1 N ammonium hydroxide

Adrenaline and noradrenaline 3 ml of the neutralized brain eluates were taken for each determination The eluates were oxidized by the addition of 0 1 ml of 0 25 per cent potassium ferricyanide solution Exactly three min later 3 ml of a mixture containing 5 N NaOH (88 %) a 2 per cent solution of ascorbic acid in water (10 %) and concentrated ethylene diamine (2 %), were added to the oxidized eluate The sample was then diluted to 10 ml with water Blanks were prepared by omitting the ascorbic acid which was added 5 min later

In the analysis of urine samples 3 ml of neutralized eluate were used to 4 ml of alkaline ascorbate EDA mixture For extracts of adrenals 1 ml of the diluted extract pH 6 3 was used with 1 ml of the mixture This mixture remains stable for 3 to 4 hours (EULER and LISHAJKO 1961)

The noradrenaline and adrenaline values were calculated by solving the equations

$$m = yN_a + xA_a$$
$$n = yN_b + xA_b$$

In these equations x and y represent the amounts of adrenaline and noradrenaline respectively m and n were the fluorimetric readings (corrected for blanks) for filter sets a and b respectively A_a A_b N_a and N_b were the values for the fluorescence per μg on filter sets a and b for adrenaline and noradrenaline respectively

Dopamine The determination of dopamine was made according to CARLSSON and WALDECK (1958) Some of these investigations were carried out on the second eluate from columns of Dowex 50 containing mainly dopamine The presence of noradrenaline in eluates from alumina did not interfere This is in agreement with CARLSSON and WALDECK (1958) and LEDUC (1961)

Adrenaline in the brain

The relative adrenaline content of mammalian brains is small and its presence has even been questioned This problem was the subject of a special study (GUNNE 1962 c) A total chromatographic separation of adrenaline from the other catecholamines was undertaken by the method of KIRSHNER and GOODALL (1957) using the weak cation exchange resin Amberlite IRC 50 The considerable losses in the procedure were compensated for by pooling a large number of brains In spite of this 4 per cent of adrenaline (of the sum of noradrenaline and adrenaline) was the lowest detectable amount Adrenaline concentrations above 4 per cent were found in brains of rats (4 5 per cent) and pigs (7 5 per cent) Later an improved separation technique using the strongly acid cation exchange resin Amberlite CG 120 (HAGGENDAL 1962) was adopted The use of this resin permitted the detection of even smaller amounts of adrenaline The adrenaline concentration in dog brains was found to approximate 2 to 3 per cent of the sum of adrenaline and noradrenaline (unpublished results)

It was noticed when brain eluates were assayed on the Coleman fluorimeter that negative adrenaline values were often obtained in eluates from the telencephalon of dogs This was probably due to some interfering substance in the brains

By separation of brain eluates from dogs on Amberlite CG 120 a fraction was obtained which gave higher readings with the a filters than with the b filters (samples treated by the THI method) A substance with those properties might obscure the presence of small amounts of adrenaline

Considering all the implications involved in the estimation of the small

against solutions containing the fluorophores of noradrenaline and adrenaline A recalibration was required every 2 to 3 weeks

Using these filter sets the fluorescence of noradrenaline is approximately equal with *a* and *b* filters while the fluorescence of adrenaline is about three times higher with *b* filters compared with *a* filters

A slow gradual change was noticed in the sensitivity of the fluorimeter probably as a result of changes in the transmission qualities of the filters It was found that this gradual change followed a pattern which was to a certain extent predictable It implied an inherent tendency of the instrument to obtain an increased sensitivity to adrenaline If the plexi glass standard was not checked against catecholamine standards for some interval the values were biassed in the direction of too high adrenaline figures while the noradrenaline values were still approximately correct

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The noradrenaline and adrenaline values were calculated by solving the equations

The influence of pH on the stability of 5 hydroxytryptamine in perchloric acid extracts was studied in the following way Nine rat brains were pooled and extracted with 90 ml 0 4 N perchloric acid (six volumes) The extract was homogenized immediately, centrifuged and filtered The resulting pH was approximately 0 5 The extract was then divided into nine equal samples four of which were adjusted to pH 4 by 2 N KOH and the perchlorate was spun down One sample was assayed immediately (starting level) Eight samples (three at pH 4 and five at pH 0 5) were stored at − 30° C The samples were taken out at different time intervals thawed and assayed for 5 hydroxytryptamine (Fig 2)

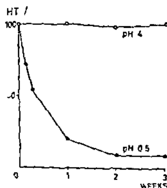


Fig 2 Stability of 5 hydroxytryptamine (5 HT) in 0 4 N perchloric acid extract of rat brains (pH 0 5) Part of the pooled extract was adjusted to pH 4 before storage at − 30 C

Results From the figure it is evident that the extracts can not be stored in the strongly acid extraction fluid without considerable losses An approximate reduction of 50 per cent was found after two days 5 Hydroxytryptamine was found to be stable at pH 4 (*cf* The Merck Index Ed VII p 932) This study shows that the rapid loss at pH 0.5 can not be prevented by freezing

For this reason all extracts intended for 5 hydroxytryptamine determination were adjusted to pH 4 by 2 N KOH before storage

Modification introduced in estimation of catecholamines and 5 hydroxytryptamine in brain extracts

In summarizing the data presented above the following modifications were introduced

Extraction was performed according to BERTLER *et al* (1958), with 0 4 N perchloric acid Immediately after centrifugation and filtration of the brain homogenate the pH of the extract was adjusted to 3 5—4 0 in

amounts of adrenaline in brain tissue, it was not thought feasible to study changes in brain adrenaline in the present investigation

3 Estimation of 5-hydroxytryptamine and 5-hydroxy-indoleacetic acid (5-HIAA)

5 hydroxytryptamine was estimated in the brain stem of dogs and total brain of rats 5-HIAA was estimated in the urine of dogs and rats

The brain content of 5 hydroxytryptamine was determined according to a method described by BERTLER (1961) Extraction was made with six volumes of 0 4 N perchloric acid After homogenization the extract was centrifuged and filtered 10 ml of the extract of dog brain stem were taken for purification and assay The total extract (approximately 10 ml) of single rat brains were treated the same way

The method of purification and assay is briefly presented here

Method of BERTLER *(1961)*

The extract was purified on a column 6 × 25 mm of the weak cation exchange resin Amberlite XE 64 After decantation of the smallest particles the columns were prepared and washed with 2 N hydrochloric acid redistilled water 20 ml of N sodium acetate buffer at pH 6 5 and finally a few ml of redistilled water

The extract was adjusted to pH 6 5 by 2 N potassium hydroxide and the perchlorate spun down at 0° C The sample was then passed through the column After washing with 10 ml of a 0 02 M phosphate buffer pH 6 5 containing 0 2 % EDTA followed by a few ml of redistilled water elution was performed by 3 ml of 1 2 N hydrochloric acid Concentrated hydrochloric acid (0 8 ml) was added to the eluate and the fluorescence was read in an Aminco Bowman spectrophotofluorimeter at 295 nm activating and 550 nm fluorescence wavelengths

5 HIAA in urine was determined according to UDENFRIEND WEISSBACH and CLARK (1955) with the modification of McFARLANE *et al* (1956)

Stability of 5 hydroxytryptamine in brain extracts

BERTLER (1961) had noticed that the immediate centrifugation of the homogenate was required in order to preclude losses of 5 hydroxytryptamine It was suggested that the amine might be adsorbed to insoluble tissue residues However, in the present study it was found that 5 hydroxytryptamine was lost whether the extract had been centrifuged or not

The influence of pH on the stability of 5 hydroxytryptamine in perchloric acid extracts was studied in the following way Nine rat brains were pooled and extracted with 90 ml 0 4 N perchloric acid (six volumes) The extract was homogenized immediately, centrifuged and filtered The resulting pH was approximately 0.5 The extract was then divided into nine equal samples four of which were adjusted to pH 4 by 2 N KOH and the per chlorate was spun down One sample was assayed immediately (starting level) Eight samples (three at pH 4 and five at pH 0 5) were stored at − 30° C The samples were taken out at different time intervals thawed and assayed for 5 hydroxytryptamine (Fig 2)

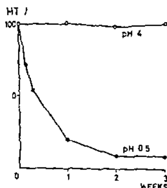


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Table I Recovery Pooled extracts or urine was divided into 8 equal parts Known amounts of noradrenaline (NA) adrenaline (A), dopamine (DA) 5 hydroxytryptamine (5 HT) or 5 hydroxyindoleacetic acid (5 HIAA) were added to six samples in each experiment

Tissue (or urine)	Volume of extract or urin ml	Substance added	Amount added μg/sample	Per cent recovered Mean	Per cent recovered Range
Dog telencephalon	40	NA	1—2	98	89—109
	40	A	1—2	76	72— 80
	40	DA	1—2	80	75— 90
Dog brain stem	15	NA	1	83	77— 92
	15	A	0 5—1	70	63— 75
	10	5HT	1	88	83— 91
Rat brain	20	NA	1—2	89	83— 97
	20	A	0 5—2	77	76— 87
	20	DA	1—2	86	82— 92
Dog urine	25	NA	1—2	71	64— 76
	25	A	1—2	68	62— 74
	5	5HIAA	50—100	101	86—120
Rat urine	25	NA	1—2	82	75— 85
	25	A	0 5—2	65	55— 71
Rat adrenals	0 5	NA	0 5—1	98	93—105
	0 5	A	0 2—0 5	99	94—105

the adrenals was expressed as μg/rat and the amounts excreted in the urine as μg/rat/h

Mean standard deviation (S D) and standard error of the mean (S E M) were computed according to ordinary formulae (FISHER 1936) Significance of means and differences were tested by the t test (FISHER 1936) Levels of significance

5 per cent level P = 0 05—0 01 probably significant*
1 per cent level P = 0 01—0 001 significant**
0 1 per cent level P < 0 001 highly significant***

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CHAPTER III

GENERAL BEHAVIOR OF DOGS, RATS AND CATS IN MORPHINE TOLERANCE AND WITHDRAWAL

1 General observations

Acute effects of morphine

In *dogs* administration of 3 mg/kg of morphine generally induced sedation or sleep from which the animals could be briefly aroused The sedation was occasionally preceded by some degree of motor unrest in combination with vomiting The sleeping time after the first morphine injection sometimes amounted to six hours, but signs of sedation could generally be observed for twelve to twenty four hours No postnarcotic excitement was seen in this species

When *rats* were given a single injection of 20 mg/kg of morphine, there was an initial sedation consisting of a more or less abolished spontaneous activity The condition resembled catalepsy with an increased muscular tone and tendency to remain in peculiar postures A partial Straub phenomenon was present, the tail was lifted and rigidly extended This stage was succeeded by one of increased activity Five to six hours after the injection no further effect could be observed in the rats

Cats given 5 to 30 mg/kg of morphine were always excited, with dilated pupils and a greatly increased rather incoordinated activity The animals were restlessly moving, jumping, rolling on the floor, etc In some cases general convulsions occurred, sometimes fatal Some degree of unrest was generally noted for more than twelve hours and was occasionally present even twenty four hours after the administration of morphine

Long term effects of morphine

In *dogs* repeated administration of morphine once daily was followed by a gradually diminished and shortened sedative effect When the dose was raised there was an increased sedation, but three weeks after the start of injections, the drug did not produce sleep in spite of any increment in dosage A complete tolerance to the emetic action was obtained within ten

days After this no vomiting was noticed as long as morphine was given Salivation was a common finding during the initial two to four weeks of morphine administration It disappeared in some dogs only to return in connection with abstinence After one to two months of morphine administration some dogs became irritable and fought frequently This was regarded as a sign of physical dependence since it occurred only at the end of the twenty four hours between injections and disappeared when the interval was shortened or the dose was raised Throughout the experiments morphine had a sedative action in dogs and no excitement was seen from the injections

The *rats* were given morphine according to two different dosage schedules (p 14) producing different behavioral effects

The regular dosage regimen with morphine injections twice daily, resulted in a successively shorter stage of sedation after each injection and an increasingly dominant stage of excitement After a week on a constant dose every injection was followed by excitement with increased activity, hyperpyrexia and exophthalmos When the dose was increased, stepwise the signs of sedation reappeared during the first few days of the new dose The same pattern of increasing excitement was then repeated

The intense dosage regimen with morphine injections at four hour intervals modified the behavior of the rats The signs of excitation were not as obvious as with the regular dosage regimen The rats appeared normal but occasional outbursts of increased activity were seen The majority of the rats gained weight and appeared healthy but sudden deaths occurred from convulsions

Cats were given morphine for five days No tolerance to the exciting effect was noticed during these days

Withdrawal and nalorphine induced abstinence

When the chronic morphine administration was withdrawn or the regular morphine injections were substituted by nalorphine a more or less pronounced abstinence excitation was noticed in *dogs* The maximal symptoms were seen approximately three days after withdrawal and all signs of abstinence had disappeared after eight to ten days Following nalorphine administration to morphine tolerant dogs there was a short lasting state of excitement with a maximal intensity of symptoms one hour after the injection After another hour the symptoms had usually disappeared The pattern of symptoms is described in detail below

In *rats* the withdrawal or nalorphine reaction was not a purely excitatory one. The condition observed after abrupt withdrawal was characterized

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In *rats* the withdrawal or nalorphine reaction was not a purely excitatory one The condition observed after abrupt withdrawal was characterized

by a combination of drowsiness with eyelid ptosis and reduced muscular tone and irritability with hyperalgesia The animals were difficult to handle, shrieking when lifted by the tail and often trying to bite During the day time they were lying rather immobile with their eyes closed, but during the night they fought frequently Piloerection, tremor and diarrhea were present and the animals lost weight The symptoms were most marked on the second day of withdrawal and disappeared within eight to ten days Nalorphine induced corresponding signs lasting for two to three hours Following the intense dosage regimen the abstinence signs became more profuse, but the general pattern of symptoms was the same and no clearcut excitation was noticed

2 Tolerance studies in rats

Spontaneous running activity

Methods The activity cages of DEREK and WANG (1926) were used These consist of a small living compartment, where the animals can rest, and a revolving drum by which the spontaneous running activity is recorded

After an acclimation period of two weeks on saline injections, five rats were given chronic morphine treatment according to the standard regimen (20—180 mg/kg twice daily) The cages were artificially illuminated from

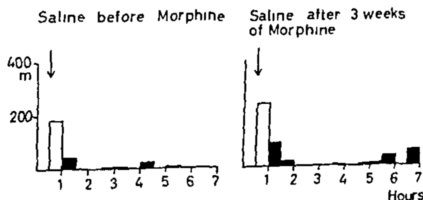


Fig 3 Spontaneous running activity of rats during light hours expressed as distance run in meters (mean of five animals) Arrows indicate saline injections White columns arousal activity after injections Black columns activity during the following six hours Left figure activity before morphine Right figure activity 24 hours after the last morphine injection

8 a m to 5 p m Cleaning of the cages was performed daily between 4 and 5 p m The room temperature was between 20° and 22° C

Results Fig 3 illustrates the unspecific arousal activity during the first half hour after the saline injections (white columns), followed by a relatively calm period during the light hours This pattern of activity was regularly repeated during the control period before the morphine injections were started (left in Fig 3) and was rapidly resumed after withdrawal of morphine (right in Fig 3)

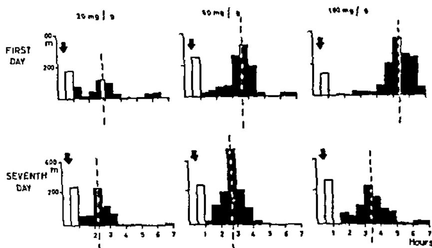


Fig 4 Spontaneous running activity of rats during chronic morphine administration For explanation of symbols consult Fig 3 Doses indicated above Upper figures activity on first day of each morphine dose level. Lower figures activity on the last day of corresponding dose levels Broken vertical lines median values of activity (white columns excluded)

During administration of morphine the activity was increased for four to six hours after each injection (Fig 4) For the purpose of calculation the first half hour of unspecific injection activity (white column) was excluded and the following six hours were studied The median values (representing the time when half of the six hours activity had been produced) were reached later on the first day of a certain dose level (Fig 4 upper figures) than on the last day of the same dose (Fig 4 lower figures)

These records show that morphine induced an increase of activity (excitation) in rats and that this activity appeared earlier with increasing tolerance

Tolerance to toxic doses

The approximate LD₅₀ for the rats was 60 mg/kg, which is unusually low. In order to ascertain to what extent the tolerance to toxic doses could be increased, the following investigation was undertaken.

Methods. Ninety two (male) rats weighing 140—170 g at the start of the experiment were used. Ten were left untreated for three weeks (control animals) while eighty two rats were made morphine tolerant by the standard dosage regimen (20—180 mg/kg twice daily). On the twenty first day, with an average weight of 220 g, they received 200 mg/kg in one dose. The controls of an average weight of 240 g, were also given one injection of morphine 200 mg/kg.

Results. The mortality in the controls receiving morphine 200 mg/kg was 80 per cent (eight rats). Of the experimental group 12 per cent (ten rats) died. Nine of these died during the three weeks of pretreatment, prior to the challenging dose of 200 mg/kg (six rats succumbed even to the initial injection of 20 mg/kg). — In later long term experiments the mortality rate was about the same (10 to 12 per cent) when the final dose was raised to 300 mg/kg. — These experiments show that the rats had acquired a relative tolerance to otherwise toxic morphine doses.

Drug combinations

When control rats received an i.p. injection of the monoamine oxidase inhibiting substance nialamid in a dose of 300 mg/kg they demonstrated drowsiness from which they could be briefly aroused, depression of the righting reflex and eyelid ptosis. This condition lasted for one to two hours and was succeeded by a stage of alertness, increased motor activity and exophthalmos, lasting for more than twenty four hours.

When the same dose of nialamid was given to morphine tolerant animals four hours after the last morphine injection, the same symptoms appeared. The initial period of somnolence in these animals was, however considerably longer, generally lasting for three to five hours. After this time the usual alertness appeared.

Following administration of 5 mg/kg i.p. of reserpine there were no noticeable differences between controls and morphine tolerant rats. This drug had a sedative action in both groups of rats.

3 Abstinence signs in dogs

Rating scale

The signs of withdrawal were rated mainly according to PLANT and PIERCE (1928) and grouped into three different grades The rating scale was well applicable also in nalorphine induced abstinence

Grade I Degree of abstinence SLIGHT

a) *Constant symptoms*

Animals friendly and quiet Stiff awkward gait fine tremors retracted belly

b) *Common symptoms*

Sleepiness moderate salivation diarrhea

c) *Occasional symptoms*

Vomiting

Grade II Degree of abstinence MODERATE

a) *Constant symptoms*

Animals restless but not continuously moving Tremors rigidity with spastic gait Vomiting Gnawing at objects

b) *Common symptoms*

Salivation Pilo-erection Twitching of muscles Diarrhea

c) *Occasional symptoms*

Whining (generally not noisy) Hiccough Panting Growling but handling possible

Grade III Degree of abstinence MARKED

a) *Constant symptoms*

Restless almost continuously moving Gnawing and swallowing inedible objects (for instance sawdust) Marked tremors and spastic gait Pilo-erection Salivation and vomiting sometimes profuse

b) *Common symptoms*

Hiccough diarrhea At intervals panting jerky respiration
Howling Muscular weakness

c) *Occasional symptoms*

Irritable and belligerent Very noisy

No dogs in this series showed *very marked* symptoms (grade IV) These were described by PLANT and PIERCE (1928) as raging wild animals continuously moving often with general convulsions

The ratings were made independently by two observers generally on the

day of sacrifice and many days before the estimations of monoamines were completed

Results Abrupt withdrawal generally gave less severe symptoms than nalorphine On withdrawal eight out of ten dogs showed slight (grade I), one moderate (grade II), and one marked symptoms (grade III) In twenty two dogs subjected to nalorphine induced abstinence seven were grade I, nine were grade II and six were grade III No convulsions were observed in these experiments

There did not appear to be any correlation between the addicting dose and the grade of induced abstinence symptoms, and there were great individual variations between dogs treated similarly Some breeds of dogs (for example beagles) appeared to develop more intense symptoms than others

4 Discussion

The depressant action of acute morphine administration in dogs and rats, as well as the excitation in cats are in agreement with earlier observations (KRUEGER, EDDY and SUMWALT 1941 p 8) A difference between the species was found to exist also with regard to the duration of action, four to six hours in rats but twelve to twenty four hours in dogs and cats

Chronic morphine treatment produced the well known tolerance phenomena in dogs and rats, *viz* a shortening of the duration and a decrease in intensity of the sedative action In rats, but not in dogs, the depression was converted into a state of excitation The exciting effect of chronic morphine administration in rats was reflected in the activity cages Earlier activity studies in rats (TONINI MISERI and BARBINI 1959 and OGURI 1961) seem to have given comparable results — No tolerance to the exciting effects of morphine was noticed in the cats, in agreement with the findings of earlier workers using doses within the same range (*cf* KRUEGER *et al* 1941, p 69)

Recently MULL and WOODS (1962) reported that the rate of disappearance of free labeled morphine was faster in the brain and blood of morphine tolerant dogs, as compared with nontolerant animals AXELROD (1956) demonstrated an enzymatic N demethylating system in liver microsomes of rats, which interacted with morphine during chronic administration These findings support the view that there is less morphine available at receptor sites in the morphine tolerant organism, but opinions differ concerning their significance for tolerance phenomena (TAKEMORI and MANNERING 1958, HERKEN, NEUBERT and TIMMLER 1959)

The diminished duration of morphine action in dogs which occurred in a later stage of the addiction cycle necessitated the introduction of two daily injections In order to produce dependence on a narcotic drug (and consequently abstinence phenomena) the intervals between injections should not be extended beyond a certain limit (*cf* REYNOLDS and RANDALL 1957 p 111) The proper time interval seemed to vary between species, more frequent doses being necessary for rats than for dogs This might reasonably be attributed to differences in the rate of destruction of the drug

Abrupt withdrawal and injection of nalorphine produced an abstinence syndrome in dogs and rats (cats were not studied) In dogs abstinence produced a clearcut excitation while rats exhibited a mixed syndrome (drowsiness and irritability) In both species there were several autonomic manifestations piloerection tremor, etc.

It has been pointed out (PLANT and PIERCE 1928, SEEVERS 1936) that a fully developed excitatory syndrome of morphine abstinence with great similarities to that of human addicts can be regularly produced in higher animals like dogs and monkeys

As far as the abstinence syndrome in rats is concerned, opinions differ According to some authors (SOLLMAN 1924 MEYERS 1931 KAYMAKCALAN and WOODS 1956 MAYNERT and KLINGMAN 1962) the abstinence symptoms in this species are either absent or can be described as a sedation Others have noticed signs of irritability and restlessness (JOEL and ETTINGER 1926 BARLOW 1932 HIMMELSBACH GERLACH and STANTON 1935 DETRICK and THIENES 1941 GUNNE 1961)

Recently a clearcut excitatory abstinence syndrome was reported also in rats (HANNA 1960 WEEKS 1962) and mice (MAGGIOLO and HUIDOBORO 1962) when morphine had been administered more or less continuously

These findings were only partly confirmed in the present study Following an intense schedule of morphine administration in rats (essentially according to the method of HANNA 1960) the abstinence signs were more pronounced than usual but not purely excitatory

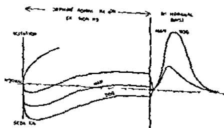


Fig 5 Schematic representation of the central nervous system excitability in different species during continuous morphine administration with increasing dose (between the vertical lines time intervals of weeks months) and after abrupt withdrawal (to the right time intervals of days)

Fig 5 is a schematic illustration of the behavior described in different species, during a hypothetical continuous administration of morphine with gradually increasing doses and after abrupt withdrawal The corresponding curve for man was drawn essentially according to SEEVERS (1954) but a minor modification was introduced, since it was felt that the response to the drug in the morphine tolerant addict is rather one of slight stimulation than of sedation (unpublished observations) The gradual change of morphine action on the excitability of the central nervous system, thus places man between the dog (which is not excited as long as morphine is given) and the rat (which becomes excited rather soon following the initial sedation) — The excitement produced by morphine administration is complex and in many respects incommensurable with the excitement of withdrawal (ISBELL and FRASER 1950), but Fig 5 may illustrate the approximate species differences also with regard to the withdrawal reaction

A few *morphine tolerance effects were studied separately* It is not generally agreed that rats acquire tolerance to toxic morphine doses (*cf* SCHAUMANN 1957, p 214) In the rats of the present study there was evidently an increasing tolerance to the toxicity of morphine Since no tolerance to the convulsive effects can be expected, this probably means that the rat strain used has been especially susceptible to the respiratory depressant *action of* the drug

The *special tolerance effects* noted in the experiment with a monoamine oxidase inhibitor, will be discussed later (p 75)

CHAPTER IX

NORADRENALINE, DOPAMINE AND 5-HYDROXY-TRYPTAMINE IN THE BRAIN IN MORPHINE TOLERANCE AND WITHDRAWAL

The effects of *acute* morphine administration on the content of noradrenaline in the brain were studied in cats and rats. Dogs and rats were used for studies of *long term* effects of morphine and *abrupt withdrawal* as well as *nalorphine induced abstinence*.

I Special methods

Some long term effects of morphine on the noradrenaline content of the rat brain were elucidated by administration of drugs interfering with the storage or metabolism of the catecholamines. The drugs were given after completion of a three weeks series of injections with saline or morphine (standard regimen, p. 14) in 112 male rats weighing 250—300 g.

All rats were sacrificed twenty four hours after the last saline or morphine injection. The rats were divided into four groups, each consisting of 12 to 14 controls and 12 to 18 morphine tolerant rats.

1) The first group (controls and morphine tolerant) was given no additional treatment.

2) The second group (controls and morphine tolerant) was given 300 mg/kg i. p. of the monoamine oxidase inhibitor nialamid (Niamid, Pfizer) four hours after the last regular morphine or saline injection (twenty hours before sacrifice).

3) The third group (controls and morphine tolerant) was given 150 mg/kg i. p. of α-methyl DOPA (Aldomet, Merck, Sharp & Dohme) four hours after the last regular morphine or saline injection (twenty hours before sacrifice).

4) The fourth group (controls and morphine tolerant) received 5 mg/kg i. p. of reserpine (Serpasil, Ciba) four hours after the last regular morphine or saline injection (twenty hours before sacrifice).

Fig 5 is a schematic illustration of the behavior described in different species, during a hypothetical continuous administration of morphine with gradually increasing doses and after abrupt withdrawal The corresponding curve for man was drawn essentially according to SEEVERS (1954) but a minor modification was introduced, since it was felt that the response to the drug in the morphine tolerant addict is rather one of slight stimulation, than of sedation (unpublished observations) The gradual change of morphine action on the excitability of the central nervous system, thus places man between the dog (which is not excited as long as morphine is given) and the rat (which becomes excited rather soon *following the initial* sedation) — The excitement produced by morphine administration is complex and *in many respects incommensurable with the excitement of withdrawal* (ISBELL and FRASER 1950), but Fig 5 may illustrate the approximate *species differences also with regard to the withdrawal reaction*

A few morphine tolerance effects were studied separately It is not generally agreed that rats acquire tolerance to toxic morphine doses (*cf* SCHAUMANN 1957, p 214) In the rats of the present study there was evidently an increasing tolerance to the toxicity of morphine Since no tolerance to the convulsive effects can be expected, this probably means that the rat strain used has been especially susceptible to the respiratory depressant action of the drug

The special tolerance effects noted in the experiment with a monoamine oxidase inhibitor, will be discussed later (p 75)

Table IX Noradrenaline (NA) content in the whole brains of rats (means ± S E M) Chronic morphine treatment was given for 3 weeks up to a final dose of 300 mg/kg given 6 times daily at 4 hours intervals (1 800 mg/kg/day) The rats were killed 4 hours after the last injection of saline or nalorphine with the exception of the withdrawal group Two brains pooled for each determination (cf Table XV for adrenals of same rats)

Number of rats	Treatment	NA µg/g
10	Saline	0 53 ± 0 012
10	Nalorphine 10 mg/kg	0 56 ± 0 009
12	Chronic morphine + saline 1 inj	0 61 ± 0 015
12	Chronic morphine + nalorphine 10mg/kg	0 60 ± 0 017
6	Chronic morphine + withdrawal 48 h	0 61 ± 0 016

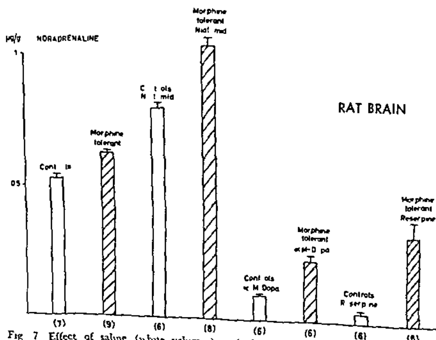


Fig 7 Effect of saline (white volumns) and chronic morph ne treatment (shaded columns) on noradrenaline content of rat brains (means + S E M) Controls and morphine tolerant rats were given nalam d 00 mg/kg α methyl DOPA 150 mg/kg or reserpine 5 mg/kg twenty hours before sacrifice Number of observations within brackets (one observation = two pooled rat brains)

Table VIII Noradrenaline (NA) and dopamine (DA) content in dog brain (means ± S E M) Nalorphine was given 5+5 mg/kg at 2 hours intervals and animals were sacrificed 2 hours after the second injection Chronic morphine (Mo) treatment was given for 70—90 days final dose 90—120 mg/kg Animals sacrificed 20 hours after last injection except in withdrawal group

Number of dogs	Treatment	Total brain NA μg/g	Brain stem NA μg/g	Cerebellum NA μg/g	Telencephalon NA μg/g	Telencephalon DA μg/g
10	None	0 16 ± 0 009	0 37 ± 0 015	0 11 ± 0 010	0 13 ± 0 009	0 20 ± 0 013
6	Nalorphine	0 17 ± 0 014	0 34 ± 0 023	0 11 ± 0 017	0 14 ± 0 011	0 24 ± 0 020
11	Chronic Mo	0 17 ± 0 008	0 35 ± 0 010	0 12 ± 0 012	0 15 ± 0 009	0 19 ± 0 012
2	Chronic Mo+ withdrawal 72 h	0 08	0 23	0 03	0 06	0 12
16	Chronic Mo+ nalorphine	0 10 ± 0 009***	0 21 ± 0 020***	0 06 ± 0 004***	0 08 ± 0 008***	0 13 ± 0 014

Different from chronic Mo P < 0 01
* Different from chronic Mo P < 0 001

Table IX Noradrenaline (NA) content in the whole brains of rats (means ± S E M) Chronic morphine treatment was given for 3 weeks up to a final dose of 300 mg/kg given 6 times daily at 4 hours intervals (1 800 mg/kg/day) The rats were killed 4 hours after the last injection of saline or nalorphine with the exception of the withdrawal group Two brains pooled for each determination (cf Table XI for adrenals of same rats)

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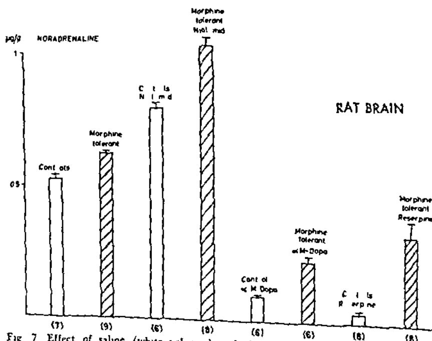


Fig 7 Effect of saline (white columns) and chronic morphine treatment (shaded columns) on noradrenaline content of rat brains (means + S E M) Controls and morphine tolerant rats were given nialamid 300 mg/kg α methyl DOPA 150 mg/kg or reserpine 5 mg/kg twenty hours before sacrifice Number of observations within brackets (one observation = two pooled rat brains)

Long term administration of morphine

No changes in the content of noradrenaline were found in the investigated parts of the *dog* brain as a result of long term morphine administration as long as the animals were morphine satiated (Table VIII). There were similarly no changes in the content of dopamine in the telencephalon or of 5-hydroxytryptamine in the brain stem (Fig. 8).

In *rats* the brain content of noradrenaline was increased both when the regular dosage regimen was applied (Fig. 7, left) and following the intense dosage regimen (Table IX).

Table X. 5-Hydroxytryptamine (5-HT) content in brain stem of dogs (means ± S.E.M.). Nalorphine was given 5+5 mg/kg at 2 hours intervals and animals were sacrificed 2 hours after the second injection. Chronic morphine (Mo) treatment was given for 70—90 days, final dose 90—120 mg/kg. Animals sacrificed 20 hours after last injection except in withdrawal group

Nr of dogs	Treatment	5-HT μg/g
10	None	0.57 ± 0.034
4	*Nalorphine*	0.56 ± 0.076
6	Chronic morphine	0.52 ± 0.031
2	Chronic morphine + withdrawal 72 h	0.55
8	Chronic morphine + nalorphine	0.54 ± 0.020

Table XI. 5-Hydroxytryptamine (5-HT) content in whole brains of rats (means ± S.E.M.). Morphine or saline given twice daily. The animals were sacrificed 4 hours after the last injection, except in the withdrawal group

Nr of rats	Treatment	Final dose mg/kg	5-HT μg/g
9	Saline 21 days	—	0.43 ± 0.033
5	Morphine 10 days	60	0.44 ± 0.091
7	Morphine 21 days	180	0.46 ± 0.087
10	Morphine 21 days + Withdrawal 48 h	180	0.42 ± 0.063

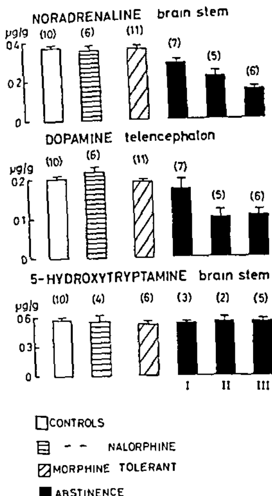


Fig 8 Effect of morphine tolerance and abstinence produced by nalorphine or abrupt withdrawal on the monoamine content in the dog brain (means + S E.M) The abstinence group (black columns) was subdivided according to severity of abstinence signs I slight II moderate and III marked Number of animals within brackets

Fig 7 illustrates the effect of drug combinations in morphine tolerant rats and controls The monoamine oxidase inhibitor nialamid produced an elevation of the noradrenaline level in controls but in morphine tolerant rats there was a significantly larger increase of noradrenaline ($P < 0001$) Alpha methyl DOPA effected a decrease of the noradrenaline level in controls but in morphine tolerant rats the decrease was smaller ($P < 0001$)

The same finding was made when the noradrenaline stores were depleted by reserpine. The depletion was significantly smaller in morphine tolerant rats than in controls ($P < 0.001$).

As in dogs, the brain content of 5-hydroxytryptamine in rats was unaltered from chronic morphine administration (Table XI).

Abrupt withdrawal

In two *dogs* the withdrawal from chronic morphine administration for 72 hours resulted in abstinence syndromes of grades II and III. The noradrenaline and dopamine values of these dogs were low (Table VIII), while 5-hydroxytryptamine was unaltered (Table X).

In *rats* the withdrawal for 48 hours did not affect the brain level of noradrenaline (Table IX) or of 5-hydroxytryptamine (Table XI). The abstinence syndrome in the rats was not purely excitatory, but was characterized by a mixture of drowsiness and irritability.

Nalorphine induced abstinence

In *dogs* given chronic morphine administration the injection of nalorphine gave rise to abstinence phenomena of various intensity, while in controls there were no obvious effects of nalorphine. The brain noradrenaline of nalorphine injected controls did not differ significantly from untreated controls or morphine tolerant dogs. The abstinence group was subdivided according to the severity of the abstinence phenomena (Grade I—III). In each of these groups there was a significant reduction of the brain stem content of noradrenaline compared with the morphine tolerant group with no signs of abstinence (Fig. 8). The reduction of noradrenaline was 21, 41 and 59 per cent in abstinence of grade I, II and III respectively. The severity of abstinence seemed to be related to the magnitude of the depletion in all parts of the brain. In the telencephalon and cerebellum, however, the relative noradrenaline decrease was even more marked at lower degrees of abstinence (grades I and II) than in the brain stem (Fig. 9).

The dopamine content of the telencephalon was unchanged in controls given nalorphine, but was reduced in connection with abstinence of grade II and III. However, when the abstinence was slight (grade I), there was no significant reduction of the dopamine content (Fig. 8).

The 5-hydroxytryptamine of the brain stem remained unchanged at any degree of abstinence (Fig. 8).

In *rats* there were no changes in the whole brain content of noradrenaline following nalorphine induced abstinence (Table IX).

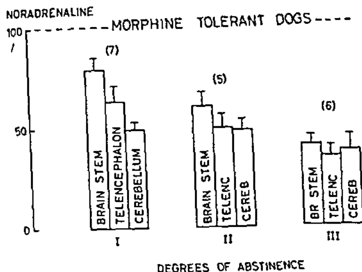


Fig 9 Noradrenaline in the brain stem telencephalon and cerebellum of dogs in various degrees of abstinence I slight II moderate and III marked abstinence signs Values expressed as per cent (means+S E M) of the corresponding levels in morphine tolerant dogs without abstinence Number of animals within brackets

3 Discussion

The *control levels* of catecholamines and 5 hydroxytryptamine in the brains of dogs rats and cats were in agreement with those reported from other laboratories (for instance BERTLER and ROSENGREN 1959 UDENFRIEND 1962 p 158) The observation of increasing catecholamine concentrations in the developing rat brain (preliminary report in GUNNE 1962 a) is in agreement with the report of KARKI KUNTZMAN and BRODIE (1962)

Seasonal variations in the catecholamines of rat brains were reported by MONTAGU (1956 b) and BEAUVALLET FUGAZZA and SOLIER (1961) These observations were not confirmed in the present study

The *acute* administration of morphine has been reported to produce various effects on the brain content of noradrenaline VOGT (1954) and QUINN *et al* (1958) obtained a decrease in the cat brain in agreement with the present results GUNNE (1959) reported a decrease in rat brains following 30 mg/kg and an increase from higher doses (60—90 mg/kg) FREEDMAN FRAM and GIARMAN (1961) administered 20 to 60 mg/kg of morphine in rats and found a decrease of noradrenaline after four hours

followed by an increase after 24 to 48 hours MAYNERT and KLINGMAN (1962) noticed only a decrease of noradrenaline in dog brains from 200 mg/kg and in rat brains from 60—200 mg/kg, while SLOAN *et al* (1962 b) found an increase in the rat brain after a morphine dose of 60 mg/kg In monkeys, SEGAL and DENEAU (1962) reported a decrease of brain noradrenaline following 3 mg/kg of morphine, but an increase after 30 mg/kg

From these rather confusing results it may be concluded that morphine exerts a dual action on the brain content of noradrenaline, some doses producing a rise while others have a tissue depleting effect Experiments on long term administration of morphine have supplied evidence that morphine acts by accelerating the synthesis of brain noradrenaline Such an action from single injections also, might account for the elevated brain levels obtained in some experiments The depletion of noradrenaline in other experiments indicates a lag of resynthesis behind the utilization, when excessive demands are made by the stimulated tissue This is generally associated with more or less obvious signs of excitation and is consequently more readily produced in cats than in rats

A lack of effect of analgesics" (PLETSCHER, SHORE and BRODIE 1956 a) and of acute morphine administration on the brain content of 5 hydroxytryptamine has been reported in rabbits (BRODIE, SHORE and PLETSCHER 1956), rats and dogs (MAYNERT, KLINGMAN and KAJI 1962) The absence of an effect on 5 hydroxytryptamine together with a depletion of brain catecholamines represents a monoamine response similar to that seen in morphine abstinence in the dog

After administration of morphine daily for five days the noradrenaline level of the cat s brain stem returned to normal This indicates a rapid tolerance to the noradrenaline depleting action of morphine

Following *chronic* morphine administration in dogs the brain content of noradrenaline was normal, in agreement with earlier observations (MAYNERT and KLINGMAN 1962, GUNNE 1962 b) The dog brain content of dopamine and 5 hydroxytryptamine was also unaltered following chronic administration of morphine

In rats the brain noradrenaline was elevated after 3 weeks of morphine Approximately the same rise was seen whether morphine was given twice or six times daily

The species difference observed between dogs and rats with respect to the brain content of noradrenaline in chronic morphine treatment may have some relation to the differences noticed in behavior Rats were excited from chronic morphine administration while dogs were sedated Earlier FREEDMAN *et al* (1961) and MAYNERT and KLINGMAN (1962) reported

a corresponding elevation of brain noradrenaline in rats on tolerance dosage schedules

Both in dogs and rats, the chronic morphine treatment had produced tolerance to the noradrenaline depletion of the acute experiment The origin of the supranormal brain levels in morphine tolerant rats was studied further The mode of action of this tolerance phenomenon could be an interference with 1) the rate of synthesis, 2) the amine binding capacity of the tissue or 3) the rate of metabolic break-down of the brain nor adrenaline Although alternative pathways should be considered the oxidative deamination by monoamine oxidase is probably the most im portant route for the metabolic break-down of catecholamines in the brain (p 11) The monoamine oxidase system seemed to be intact in brains of morphine tolerant rats since the 5 hydroxytryptamine levels were normal

When the monoamine oxidase was blocked by a long acting inhibitor (nialamid) the brain noradrenaline increased more in morphine tolerant than in control rats MAYNERT and KLINGMAN (1962) obtained the same result with a different monoamine oxidase inhibitor (JB 516) These find ings indicate that the difference in brain noradrenaline between controls and morphine tolerant rats is not found in the rate of metabolic break down

The possibility that chronic morphine administration had acted on the noradrenaline binding capacity of the brain tissue was tested by adminis tration of two noradrenaline releasing agents α methyl DOPA and reser pine These compounds are known to reduce the noradrenaline binding capacity of brain tissue probably by different mechanisms (p 10) Follow ing administration of either of these drugs there were still noticed higher noradrenaline levels in morphine tolerant rats than in controls This in dicates that the elevated noradrenaline content in the brains of morphine tolerant rats is probably not explained solely on the basis of an increased storage capacity but rather by an increased rate of synthe is On the other hand an alteration of the catecholamine binding properties in brain tissue is not ruled out (*cf* p 75)

In experiments where combinations of drugs have been used the pos sibility of a direct interaction and/or competition at receptor sites hould also be taken into consideration That some interaction actually occurs is suggested by the finding of FREEDMAN *et al* (1961) that morphine could partially reverse the noradrenaline releasing action of reserpine in the brains of rats whether morphine was given 24 hours before or after reserpine

In connection with *nalorphine induced abstinence* there was a reduction

of noradrenaline in the brain stem of dogs This is in accordance with earlier reports (MAYNERT and KLINGMAN 1962, GUNNE 1962b)

The present study showed that the severity of the abstinence symptoms was proportional to the depression of noradrenaline This was true for all parts of the brain, but certain differences seem to exist The brain stem appeared to be more resistant to the noradrenaline reducing action compared with the cerebellum and telencephalon Only in cases of marked abstinence were all 3 parts (brain stem, telencephalon and cerebellum) equally depleted The differences found may reflect different modes of storage It is known (CARLSSON, FALCK and HILLARP 1962) that the noradrenaline of the brain stem is stored in granules within the nervous tissue The noradrenaline of cerebellum and telencephalon might be chiefly located at vasomotor nerve endings (*cf* EULER 1946, VOGT 1954), and these stores could thus be regarded as located peripherally

The fact that dopamine is also partially depleted in morphine abstinence in the dog can probably not be explained merely on the basis of an increased utilization of the precursor of noradrenaline The magnitude of the reduction in the forebrain stores of dopamine indicates that this amine is also directly involved in the release SEGAL and DENEAU (1962) obtained changes in the brain dopamine levels of monkeys at variance with those seen in dogs in the present report A decrease was noted in morphine dependence, but an increase followed abrupt and nalorphine induced withdrawal (numerical data were not presented) No explanation for this difference in results can be given at present

Following *abrupt withdrawal* from chronic morphine administration in dogs, the same general pattern of brain monoamines was noticed as in nalorphine induced abstinence a reduction of noradrenaline and dopamine, with unaltered 5 hydroxytryptamine levels

In rat brains there was no reduction of noradrenaline in withdrawal or nalorphine induced abstinence, in accordance with MAYNERT and KLINGMAN (1962) In an earlier study (GUNNE 1959) a slight decrease of noradrenaline was noted also in rats following withdrawal SLOAN *et al* (1962a) obtained a reduction of catecholamines in the heart of rats 48 hours after withdrawal from chronic morphine treatment but found no effect in the brain

Generally, the effects of morphine abstinence on brain noradrenaline in rats have been small or absent, in contrast with the findings in dogs The reason for this species difference is not clear but again it is tempting to correlate the difference with the clinical effects of withdrawal in the two species Dogs were excited, while rats showed a mixed state of drowsiness

and irritability If a noradrenaline release actually takes place in the rat brain however, it might be concealed by the rapid resynthesis in morphine tolerance

GUNNE (1961) and SLOAN *et al* (1962 a) reported that morphine withdrawal in rats did not release 5 hydroxytryptamine in the brain MAYNERT *et al* (1962) found no effect on brain 5 hydroxytryptamine following nalorphine administration in morphine tolerant dogs and rats The present investigation confirmed these observations and further showed that 5 hydroxytryptamine was unchanged irrespective of the degree of abstinence intensity

CHAPTER V

ADRENALINE AND NORADRENALINE IN THE ADRENAL GLANDS IN MORPHINE TOLERANCE AND WITHDRAWAL

The effects of *acute* morphine administration on the content of adrenaline and noradrenaline in the adrenal gland were studied in cats and rats Dogs and rats were used in studies of the effects of *long term* morphine administration and *abrupt withdrawal* as well as *nalorphine induced abstinence*

1 Special methods

Unilateral sectioning of the splanchnic nerve was performed in a few morphine tolerant dogs The animals were anaesthetized with pentobarbital (Nembutal, Abbott) 40 mg/kg i p and were intubated Via a midline abdominal incision the left splanchnic nerve was exposed immediately below the diaphragm and was cut Aseptic conditions were not observed, but 500 000 units of penicillin (Astracillin, Astra Ltd) was given daily for 1 days after the denervation The animals were sacrificed within 20 days after the denervation, and were then checked for signs of regeneration of the splanchnic nerve No gross changes in behavior were noted in the operated animals and no infections occurred

2 Results

Control levels

The adrenaline and noradrenaline content of the adrenal glands expressed as μg/pair of adrenals, increased during lifetime in the rats (Fig 10)

The same tendency was noticed also in adrenal glands of dogs of different age The material was too small, however for a statistical treatment and the difference between age groups was diminished when the catecholamine level was expressed per gram of wet tissue weight (p 28) In spite of this there was a wide range of the adrenaline content in the adrenals of untreated control dogs (and nalorphine injected controls) 0 69 – 2 88 mg/g of wet tissue weight

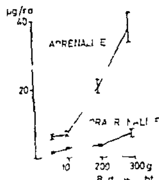


Fig 10 Adrenaline and noradrenaline content of rat adrenals calculated per pair of adrenals Means ± S E M of groups of twelve rats of different body weights Two pairs pooled for each determination

Acute and 5 days administration of morphine

Administration of a single morphine dose (30 mg/kg) gave a 70 per cent reduction of adrenaline and an 80 per cent reduction of noradrenaline in the adrenal glands of *cats* sacrificed four hours after the injection Three animals died from convulsions within 2 to 3 hours after the injection In two of these cats there was no reduction of the content of adrenaline or

Table XII Adrenaline (A) and noradrenaline (NA) mg/g tissue weight in adrenal glands of cats following a single (acute) and 5 days administration of morphine (once daily) 30 mg/kg Individual values represent means of 2 determinations (For brains cf Table XI)

	Controls		Morphine acute		Morphine acute		Morphine for 5 days	
	A	NA	A	NA	A	NA	A	NA
	0 90	0 90	1 64	2 10	0 46	0 12	0 25	0 48
	1 04	0 54	1 34	1 12	0 34	0 15	0 16	0 26
	1 03	0 70	0 28	0 04	0 36	0 09	0 21	0 23
	1 54	0 84			0 13	0 17	0 17	0 60
Means	1 13	0 75	(1 09)	(1 09)	0 32 *	0 13**	0 20***	0 39*

Died from convulsions 2—3 hours after injection.
[2] Sacrificed 4 hours after (last) injection.
* Different from controls P < 0 05
** P < 0 01
*** P < 0 001

CHAPTER V

ADRENALINE AND NORADRENALINE IN THE ADRENAL GLANDS IN MORPHINE TOLERANCE AND WITHDRAWAL

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Table VII Adrenaline and noradrenaline content and weight of adrenal glands of dogs (means ± S E M) Nalorphine was given 5+5 mg/kg at 2 hours intervals and animals were sacrificed 2 hours after the second injection Chronic morphine (Mo) treatment was given for 70—90 days final dose 90—120 mg/kg Animals sacrificed 20 hours after last morphine injection except in the withdrawal group

Number of dogs	Treatment	Weight g	Adrenaline mg/g	Noradrenaline mg/g
10	None	1.12 ± 0.11	1.37 ± 0.11	0.23 ± 0.03
6	Nalorphine	1.24 ± 0.11	1.39 ± 0.31	0.25 ± 0.03
10	Chronic Mo	1.15 ± 0.13	1.41 ± 0.10	0.25 ± 0.03
2	Chronic Mo+ withdrawal 72 h	1.30	0.22	0.09
14	Chronic Mo+ nalorphine	1.36 ± 0.11	0.60 ± 0.08***	0.15 ± 0.02

*Different from chronic Mo $P < 0.001$

(p 14) was a combination of drowsiness and irritability The noradrenaline content of the adrenals was mainly unaffected in both species

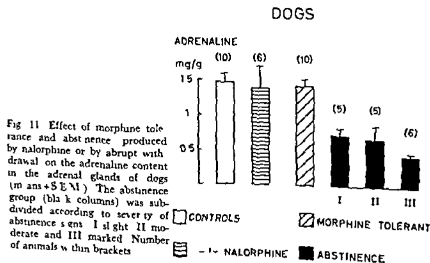


Fig 11 Effect of morphine tolerance and abstinence produced by nalorphine or by abrupt withdrawal on the adrenaline content in the adrenal glands of dogs (means + S E M) The abstinence group (black columns) was subdivided according to severity of abstinence signs I slight II moderate and III marked Number of animals within brackets

Table XIII Adrenaline and noradrenaline content in the adrenal glands of rats (means ± S E M) The rats were given one or two injections of morphine and were sacrificed 4 hours after the first injection (Second injection was given 2 hours after the first) The adrenals of two rats were pooled for each determination (For brains cf Table VII)

Number of rats	Treatment	Dose	Adrenaline µg/rat	Noradrenaline µg/rat
12	Controls, saline	0.2 ml	26.1 ± 2.0	3.5 ± 0.3
10	Morphine	20 mg/kg	11.0 ± 2.7*	2.6 ± 0.8
10	„	30 mg/kg	12.4 ± 1.8***	2.8 ± 0.6
10	„	30+30 mg/kg	3.9 ± 1.5***	1.4 ± 0.7*

* Different from controls $P < 0.05$
** „ $P < 0.01$
*** „ $P < 0.001$

noradrenaline (Tab XII) When the same morphine dose had been given daily for five days the mean adrenaline level was lower than in the acute experiment while the noradrenaline level showed a beginning return (different from acute experiment $P < 0.05$)

When morphine was given to *rats* in acute experiments there was a reduction of adrenaline with doses of 20 to 60 mg/kg, and a dose—response relationship was established (The last mentioned dose was divided in 30 + 30 mg/kg) The noradrenaline content was reduced only following the highest dose (Table XIII)

Long term administration of morphine

No changes were found in the adrenaline and noradrenaline content of the adrenal glands *in dogs* (Fig 11) *or rats* (Table XV) rendered morphine tolerant by long term administration

Abrupt withdrawal

In *dogs* an abrupt withdrawal, resulting in abstinence symptoms of grade *II* and grade III, was accompanied by a reduced adrenaline content (two animals), Table XIV

In *rats* withdrawal also caused a marked decrease in adrenaline content Forty eight hours after abrupt withdrawal the mean adrenaline content was 48 per cent of that found *in adrenals of morphine* tolerant rats (Table XV) The abstinence symptoms of these rats treated by the intense regimen

Table VII Adrenaline and noradrenaline content and weight of adrenal glands of dogs (means ± S E M) Nalorphine was given 5+5 mg/kg at 2 hours intervals and animals were sacrificed 2 hours after the second injection Chronic morphine (Mo) treatment was given for 70—90 days final dose 90—120 mg/kg Animals sacrificed 20 hours after last morphine injection except in the withdrawal group

Number of dogs	Treatment	Weight g	Adrenaline mg/g	Noradrenaline mg/g
10	None	1 12 ± 0 11	1 37 ± 0 11	0 23 ± 0 03
6	Nalorphine	1 24 ± 0 11	1 39 ± 0 31	0 25 ± 0 03
10	Chronic Mo	1 15 ± 0 13	1 41 ± 0 10	0 25 ± 0 03
2	Chronic Mo + withdrawal 72 h	1 30	0 22	0 09
14	Chronic Mo + nalorphine	1 36 ± 0 11	0 60 ± 0 08***	0 15 ± 0 02

* Different from chronic Mo P < 0 001

(p 14) was a combination of drowsiness and irritability The noradrena line content of the adrenals was mainly unaffected in both species

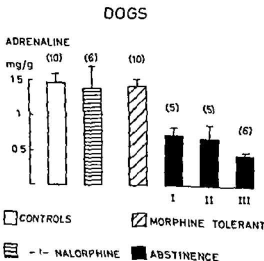


Fig 11 Effect of morphine tolerance and abstinence produced by nalorphine or by abrupt withdrawal on the adrenalin content in the adrenal glands of dogs (means + S E M) The abstinence group (black columns) was subdivided according to severity of abstinence signs I slight II moderate and III marked Number of animals within brackets

Nalorphine induced abstinence

In morphine tolerant *dogs* the injection of nalorphine induced abstinence syndromes of various degrees and a marked decrease of adrenaline in the adrenal glands The noradrenaline content was not significantly decreased In non tolerant dogs there were no behavioral effects of nalorphine and the adrenaline and noradrenaline content was normal (Table XIV) The

Table XV Adrenaline (A) and noradrenaline (NA) content in the adrenal glands of rats (means ± S E M) Chronic morphine treatment was given for 3 weeks, up to a final dose of 300 mg/kg, given 6 times daily at 4 hours intervals (1 800 mg/kg day) The rats were killed 4 hours after the last injection of saline or nalorphine with exception of the withdrawal group Adrenals of 2 rats pooled for each determination (cf Table IX for brains of same rats)

Number of rats	Treatment	A µg/rat	NA µg/rat
10	Saline	24 5 ± 1 33	4 4 ± 0 68
10	Nalorphine 10 mg/kg	22 0 ± 1 58	5 7±0 45
12	Chronic morphine + saline 1 inj	26 8 ± 1 73	4 2 ± 0 77
12	Chronic morphine + nalorphine 10 mg/kg	23 1 ± 1 86	3 7 ± 0 52
6	Chronic morphine + withdrawal 48 h	13 9 ± 1 93**	2 2 ± 0 52

** Different from chronic morphine P < 0 01

abstinence group was subdivided according to the degree of abstinence and Fig 11 shows that the largest reduction of adrenaline content occurred in animals which exhibited the most pronounced abstinence symptoms The reduction of adrenaline was 49, 52 and 68 per cent in abstinence of grades I II and III respectively

In *rats* the adrenaline and noradrenaline content was not correspondingly reduced by nalorphine (Table XV) No clearcut excitatory abstinence was seen following nalorphine in morphine tolerant rats

Unilateral splanchnic section

Results of experiments on three morphine tolerant dogs in which the *left* splanchnic nerve had been cut are shown in Fig 12 One of these dogs

was given saline and two received nalorphine 5 + 5 mg/kg with resulting abstinence signs (right in Fig 12) For comparison four non-operated dogs were included, two morphine tolerant and two morphine tolerant + nalorphine (left in Fig 12) The figure demonstrates that the adrenaline content was almost equal on the right and left side in all groups, the only exception being the abstinence group in which the nerve had been cut

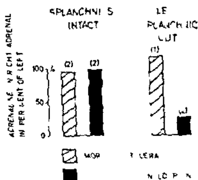


Fig 12 Adrenaline content of the right adrenal expressed in per cent of adrenaline content of left adrenal gland of dogs *Note that after denervation of the left adrenal there is a marked reduction of the adrenaline content only in the innervated (right) adrenal after nalorphine induced abstinence* Numbers of dogs within brackets

In these dogs the adrenaline level was definitely subnormal on the right innervated side (0 16 and 0 18 mg/g) in agreement with the findings reported above for morphine tolerant dogs given nalorphine In the denervated gland probably no reduction of adrenaline had occurred The values were essentially normal, although in the lower part of the normal range (0 66 and 0 84 mg/g)

3 Discussion

The *control* values for adrenaline and noradrenaline in adrenal glands of untreated dogs rats and cats are in fair agreement with earlier *reports* (*cf* EULER 1956 p 113—114) HOKFELT (1951) has given an account of the catecholamine content of rats from foetal to adult stage The present report confirms the finding of a rapid increase especially of adrenaline in the developing rat adrenal This finding emphasized the importance of using control rats preferably of approximately the same age in every long term morphine experiment

The *acute* administration of morphine to cats and rats reduced the adrenal gland content of adrenaline and noradrenaline This effect appeared after a latent period since two cats which died from convulsions 2 to 3 hours after the morphine administration had normal values for both

amines After four hours, however, all values were depressed VOGT (1954) reported a corresponding lag in the depletion of brain and adrenal catecholamines after administration of various drugs, including morphine, in cats The exact meaning of this lag time is obscure It would appear from this study that the depletion is a rapid process which takes place 3 to 4 hours after the morphine injection

After daily administration of morphine to cats for five days a beginning replenishment of adrenal noradrenaline was observed This is in accordance with the data of BUTTERWORTH and MANN (1957 a, b), and BERTLER, HILLARP and ROSENGREN (1960), who found that stimulation of the adrenal medulla resulted in an increased synthesis of noradrenaline Any stimulation producing an increased output of adrenaline would be likely to cause a rise of the resynthesis of noradrenaline in the adrenal glands This may explain the findings in dogs and rats that when adrenaline was reduced, the noradrenaline level generally remained normal In acute experiments on rats only the intense stimulation of two consecutive morphine injections also produced a decline of noradrenaline

MAYNERT and KLINGMAN (1962), in acute morphine experiments obtained a decrease in the adrenal gland content of adrenaline in dogs and rats only after higher morphine doses (200 mg/kg in dogs, 60—200 mg/kg in rats) The present study demonstrates that there are probably strain differences between rats with respect to the acute effects of morphine, since the rats of this study responded already, in the same way, to doses of 20 and 30 mg/kg of morphine

In *morphine tolerant* dogs and rats the catecholamine content was normal This finding shows that tolerance had developed to the adrenaline reducing effect of the acute experiments

When abstinence was induced by *abrupt withdrawal*, there was a marked reduction of the adrenaline content of the adrenals both in dogs and rats *Nalorphine induced abstinence* reduced the adrenaline content only in dogs in agreement with MAYNERT and KLINGMAN (1962) and GUNNE (1962 b) If the abstinence symptoms of the dogs were graded according to severity (grades I—III), it was seen that the catecholamine reduction was largest in the group with the most pronounced symptoms (grade III) Nalorphine was without effect in the adrenals of morphine tolerant rats This need not, however, be interpreted as a principal difference between dogs and rats with regard to the catecholamine response during abstinence It was noted that a depletion of adrenaline actually occurred also in rats, when the chronic morphine treatment was abruptly withdrawn, indicating that an adrenaline release was involved in both species

The mechanism of action of the adrenaline depletion in nalorphine induced abstinence in the dog was elucidated by the experiment, in which one adrenal gland had been denervated Normally the right and left adrenals contain equal amounts of adrenaline as shown in cats (BUTTERWORTH and MANN 1957 c) and dogs (GUNNE to be publ) This was found to be true in non-operated dogs both in morphine tolerance and when the adrenaline content had been depleted by abstinence Unilateral denervation did not seem to influence the adrenal gland content of adrenaline in the morphine tolerant dog without abstinence signs When nalorphine was given however a depletion was produced on the innervated side The sectioning of the splanchnic nerve on one side had probably prevented the adrenaline release in the denervated gland during nalorphine induced abstinence

This finding suggests that the depletion of adrenaline in the adrenal medulla seen in morphine abstinence is produced indirectly through the secretory nerves

Table XVI Urinary control levels of adrenaline (A), noradrenaline (NA) and 5 hydroxyindoleacetic acid (5 HIAA) in dogs and rats

Species (number of animals)	Number of observations	Excretion product	Urinary output Mean ± S E.M	(S D)
Dog (4)	24	A	7 8 ± 0 8	(4 0) ng/kg/h
	24	NA	31 6 ± 1 9	(9 3) ng/kg/h
	24	5 HIAA	6 2 ± 0 7	(3 3) μg/kg/h
Rat (20)	35	A	4 9 ± 0 22	(1 3) ng/rat/h
	35	NA	22 ± 0 80	(4 8) ng/rat/h
	9	5 HIAA	1 50 ± 0 079	(0 24) μg/rat/h

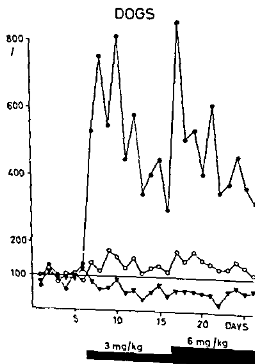


Fig 13 Mean urinary excretion of adrenaline ●——● noradrenaline o——o and 5-HIAA ▼——▼ in four dogs 100 % represents the resting level of each substance during an initial control week Black figure indicates morphine administration at two different dose levels 3 and 6 mg/kg/day

kg/h) lasting for four days After this the values tended to return and on the tenth day the rise was only three fold (Fig 13) The noradrenaline output followed essentially the same pattern but the percentage changes

were small compared with adrenaline (absolute values 36—56 ng/kg/h) The urinary 5 HIAA was diminished during morphine administration

In *rats* the same pattern of excretion was obtained for adrenaline and noradrenaline The first two daily injections of morphine, 20 mg/kg, gave a rise of both catecholamines, but one week later the same dose had a much smaller effect (Fig 14)

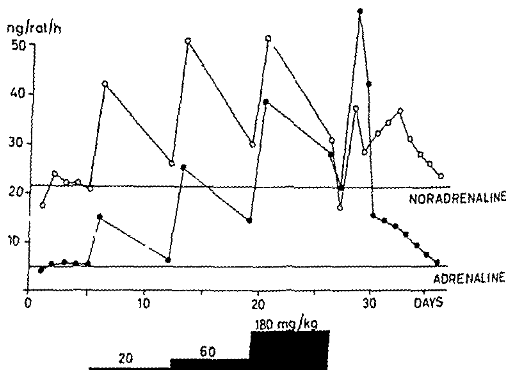


Fig 14 Urinary adrenaline ●——● and noradrenaline o——o excretion in rats before during and after a three weeks cycle of morphine Each value represents mean of three groups of rats Control levels indicated by horisontal lines Black figure morphine doses given twice daily

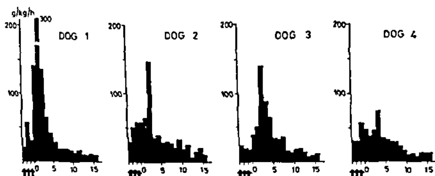
Stepwise increase of morphine dose

Whenever the morphine dose was raised, stepwise, the same pattern of an initial increase followed by another decrease reappeared in *dogs* (Fig 13) and *rats* (Fig 14) There was not, however, a complete normalization of the adrenaline output as long as morphine was given, while the noradrenaline level returned within the normal range both in dogs and rats

The 5-HIAA excretion remained low in *dogs* but was found to be elevated in *rats* following chronic morphine administration (see below)

Abrupt withdrawal

When the chronic morphine administration was abruptly withdrawn there was a rise in the urinary output of adrenaline and noradrenaline in *dogs* All four dogs showed only slight abstinence symptoms (grade I) and in no case was there any decrease of body weight or rise in body temperature (recorded daily by a thermocouple inserted 5 cm into the rectum) No major differences in symptoms could be seen between the four dogs during withdrawal but the adrenaline rise showed rather great individual variations (75—300 ng/kg/h) (Fig 15) The noradrenaline rise was more



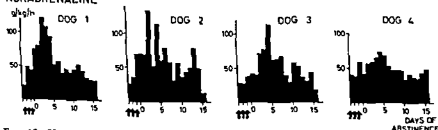


Fig 15 Urinary excretion of adrenaline (upper figures) and noradrenaline (lower figures) of individual dogs during withdrawal from chronic morphine treatment Arrows indicate the last three injections of morphine 90 mg/kg

evenly distributed between the dogs (73—135 ng/kg/h) The mean excretion curves (Fig 16) reached a maximum for adrenaline on the third day of withdrawal followed by a rapid decline The noradrenaline excretion reached a first maximum on the third day of withdrawal, simultaneously with the adrenaline maximum On the same day the abstinence symptoms were most prominent

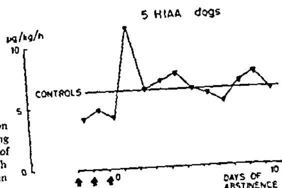


Fig 17 Mean urinary excretion of 5-HIAA in four dogs during the last three days of a cycle of addiction and ten days of withdrawal Each arrow indicates injection of morphin 90 mg/kg

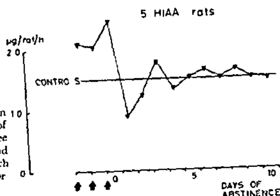


Fig 18 Mean urinary excretion of 5 HIAA in three groups of (five) rats during the last three days of a cycle of addiction and ten days of withdrawal Each arrow indicates injection of morphine 180 mg/kg

Nalorphine induced abstinence

In dogs the effect of nalorphine (10 mg/kg) was initially studied in non tolerant animals during the control period Nalorphine was without effect on the output of noradrenaline and 5 HIAA in non tolerant dogs but gave a rise of adrenaline (to 37** ng/kg/h) on the day of injection An injection of saline was without effect No symptoms were noticed following nalorphine in these dogs before morphine treatment

After chronic morphine administration nalorphine (10 mg/kg) produced a moderate abstinence syndrome (grade II) in all four dogs Fig 19 (left) illustrates the effect on the daily output of adrenaline noradrenaline and 5 HIAA When one daily morphine injection (90 mg/kg) was substituted by nalorphine there was a rise of all three excretion products Adrenaline increased nine fold on the day of nalorphine, from 29 ng/kg/h (during morphine treatment) to 260 ng/kg/h (= 25 µg/dog/24 h) When the interrupted morphine administration was continued the adrenaline rise dis

** Different from control P < 0 01

appeared again The noradrenaline rise was five fold, from 27 ng/kg/h (during morphine treatment) to 130 ng/kg/h (= 12 μg/dog/24 h) When the morphine injections were reinstituted it was found that the noradrenaline rise outlasted the adrenaline rise by two days The output of 5 HIAA was doubled on the day of nalorphine

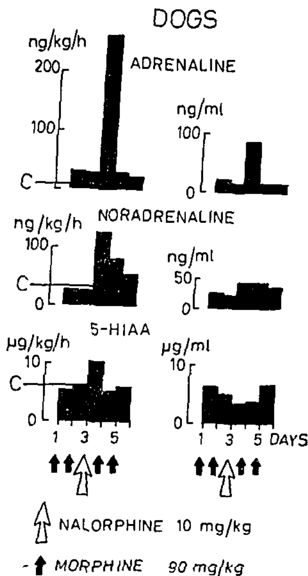


Fig 19 Left figures mean *urinary* excretion of adrenaline noradrenaline and 5 HIAA in four dogs *made* tolerant to 90 mg/kg of morphine daily by injections for two months C = control levels of *same animals before morphine treatment* On the third day of experiment the *daily morphine injections* were substituted by nalorphine 10 mg/kg Right figures urinary concentration of each substance during the same experiment

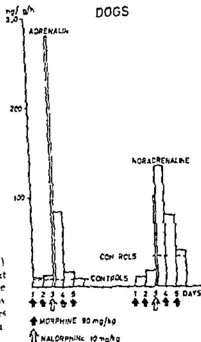


Fig 20 Urinary excretion of adrenaline (left) and noradrenaline (right) in morphine tolerant dog On the third day of the experiment the daily morphine injections were substituted by 10 mg/kg nalorphine The first four hours portion of urine following nalorphine was assayed separately

The concentration (per ml of urine) of adrenaline and noradrenaline rose as a result of nalorphine (Fig 19 right) while there was a decrease of the 5 HIAA concentration The changes in diuresis are discussed in the following section

An attempt was made to determine the sequence of events with regard to catecholamine excretion in this nalorphine experiment The urine of the first four hours following the injection of nalorphine was analyzed separately and the output of the next twenty hours in a second portion In two of the animals there was a conspicuous rise of adrenaline already in the first portion (exemplified in Fig 20) The noradrenaline rise in all animals was highest during the following twenty hours

Following nalorphine in morphine tolerant *rats* there was diarrhea and a great reduction of the urine volumes This made a satisfactory collection of urine impossible in such experiments on rats)

2 Diuresis

The effects of morphine on the urine volumes in *dogs* are illustrated in Fig 21 The daily morphine injections diminished the output of urine

appeared again The noradrenaline rise was five fold, from 27 ng/kg/h (during morphine treatment) to 130 ng/kg/h (= 12 μg/dog/24 h) When the morphine injections were reinstituted it was found that the noradrenaline rise outlasted the adrenaline rise by two days The output of 5 HIAA was doubled on the day of nalorphine

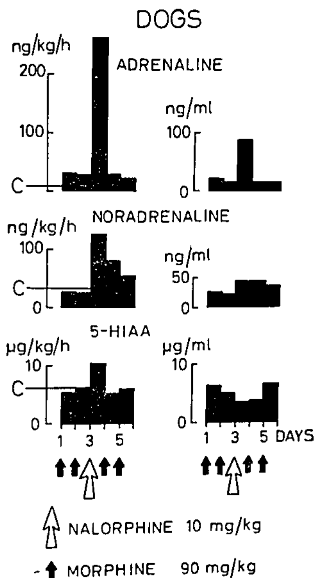


Fig 19 Left figures mean urinary excretion of adrenaline noradrenaline and 5 HIAA in four dogs made tolerant to 90 mg/kg of morphine daily by injections for two months C = control levels of same animals before morphine treatment On the third day of experiment the daily morphine injections were substituted by nalorphine 10 mg/kg Right figures urinary concentration of each substance during the same experiment

In *rats* the urine volumes were increased during chronic morphine administration as compared with saline injected controls

After withdrawal there were diminished urine volumes in the rats on the first two days of abstinence followed by another rise (Fig 22) As the rats were gaining weight during the experiments, from 160 to 250 g there was a gradual increase of urine volume also in the controls

3 Discussion

Resting levels of adrenaline and noradrenaline in the urine of rats showed a good agreement with earlier reports in which a similar technique was employed (SCHAPIRO 1958 PERMAN 1961 LEDUC 1961) The catecholamine values in dogs were considerably lower (about 1/10) than those reported by LEROY and SCHAEPDRYVER (1961) These authors used adult dogs while the present study was made on puppies

Earlier reports containing control values for urinary 5 HIAA in non anaesthetized dogs (ERSPAMER and CICERI 1957 RENSON 1960) and rats (ERSPAMER 1954 b BERTACCINI and ERSPAMER 1962) were in agreement with those of the present paper

The two male dogs excreted consistently less than half the amount of 5 HIAA compared with the two females UDENFRIEND, TITUS and WEISSBACH (1955) have pointed out that there are great individual variations in the excretion of 5 HIAA in dogs Whether there is an actual sex difference as has been reported for histamine excretion in rats (NETTER COHN and SHORE 1961) remains an open question

In non tolerant dogs and rats the *acute* injection of morphine resulted in an increased output of adrenaline and noradrenaline When the same dose had been repeated for some days the excretion of both catecholamines were showing a return towards normal levels The urinary adrenaline remained supranormal as long as morphine was given while noradrenaline decreased to normal levels after *long term* administration of the drug In both species the same cyclic pattern of excretion a rise followed by another decrease was seen whenever the morphine dose was increased stepwise

The experiments indicate that the catecholamine production and/or release are involved in the mechanisms concerned with adaptation of the organism to morphine Whether there is a decline of the initial release when the morphine injections are repeated or an increased enzymatic destruction of the free amines remains to be settled

After *withdrawal* from chronic morphine administration there was a

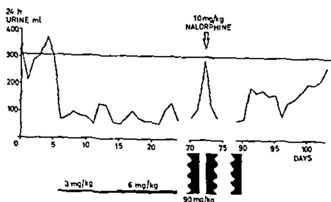


Fig 21 Daily urine volumes ml/dog/24 hours (means of four dogs) before and during chronic morphine administration (black figures daily morphine doses) Effect of nalorphine and abrupt withdrawal when the dogs had been made tolerant to 90 mg/kg of morphine Horisontal line mean control level before treatment

to about one third of the control level No obvious signs of tolerance to this effect were noticed during the ten days when the initial morphine dose was maintained

Nalorphine temporarily inversed the antidiuretic effect of morphine, thus producing a rise of the urine volumes compared with the adjacent days of morphine treatment After withdrawal there was another rise on the first day, but the urine volumes were still subnormal Following another few days of low output there was a return towards the control level

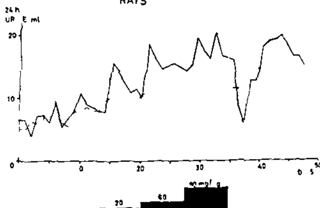


Fig 22 Daily urine volumes ml/rat/24 hours before during and after a three weeks cycle of morphine Broken line saline treated controls (one group of five rats) Solid line morphine treated (means of three groups of rats) Black figure morphine doses given twice daily

line rise by 1 to 2 days The nalorphine induced abstinence symptoms on the other hand lasted only two hours after the injection This indicates that part of the noradrenaline may originate from the adrenal glands depleted of their adrenaline stores

In all experiments on urinary excretion products in morphine administration and withdrawal fluctuations in the urine volumes may imply sources of error The findings of EULER (1956 p 167) PEKKARINEN (1961) and LEDUC (1961) indicate that the daily urinary excretion of catecholamines is largely uninfluenced by physiological variations of the diuresis Great artificial increases in the urinary flow may however bring about an increased output of catecholamines LEFOY and SCHAEPDRYVER (1961), on the other hand reported a close parallelism between the urine volumes and the output of catecholamines in dogs

In the present study it was noticed that increases in the urine volumes in dogs and rats during the control period were generally accompanied by reduced catecholamine concentrations This suggests that the daily excretion of amines was largely uninfluenced by changes in diuresis Such a view was further supported by a comparison between dogs and rats during chronic morphine treatment and withdrawal The patterns of catecholamine excretion were mainly identical although the fluctuations in diuresis were moving in opposite directions in the two species

Recently it has been shown that urinary 5 HIAA can be greatly increased in rats given water by stomach tube and that the excretion of this acid is largely dependent on the diuresis both in rats (PERTACCINI and ERSPAMER 1962) and dogs (LEWIS 1959)

The changes in urine volumes observed in dogs and rats during administration of morphine and during withdrawal are in accordance with earlier reports (the matter is reviewed by SCHAUMANN 1957 p 70) The increased intake of water and the increased urine volumes of morphine tolerant rats (BARBOUR *et al* 1929) may be connected with the fever produced by the drug in this species (GUNNE 1960)

The output of 5 HIAA was decreased in dogs and increased in rats during chronic morphine administration On the first 1 to 2 days of withdrawal these excretion patterns were reversed an increase was seen in dogs and a decrease in rats The concomitant changes in urine volumes support the view that the fluctuations in 5 HIAA were secondary to changes in the diuresis

The nalorphine induced abstinence in the dog was accompanied by a rise of both catecholamines and 5 HIAA together with an increased diuresis Despite the large urine volumes the concentration of noradrenaline and

greatly increased output of adrenaline and noradrenaline in dogs and rats. The maximal symptoms of abstinence in both species coincided with the highest adrenaline excretion and a simultaneously appearing first outburst of noradrenaline excretion. The magnitude of the adrenaline secretion was apparently unrelated to the abstinence symptoms of the four dogs. It may be assumed that the differences in adrenaline output reflects merely temporary differences in the size of the stores of the adrenal glands. A wide range was found for adrenaline in the adrenal glands of dogs. — A few days later in the withdrawal period there was a second rise of noradrenaline both in dogs and rats. By that time the adrenaline excretion was rapidly declining and the symptoms of abstinence were fading.

The first rise of adrenaline excretion, appearing simultaneously with several obvious signs of increased autonomic activity, is probably to be ascribed to a release at adrenergic nerve terminals throughout the organism. The second increase of noradrenaline may have a different explanation.

As already argued in the previous chapter (p. 58) the intense stimulation of the adreno-medullary system, effecting a depletion of the adrenaline content of the adrenal glands, elicits an increased synthesis of its precursor noradrenaline. If the adrenal gland is further stimulated after depletion it would be likely to discharge more noradrenaline than otherwise. Thus the second increase of urinary noradrenaline during withdrawal from morphine, may be derived from the partly exhausted adrenal glands.

Before the morphine administration in non-tolerant dogs a single injection of *nalorphine* only gave a small rise of urinary adrenaline (37 ng/kg/h), while noradrenaline and 5-HIAA were not affected. In morphine tolerant dogs the output of adrenaline rose to a mean of 260 ng/kg/h (controls 7.8 ng/kg/h) and noradrenaline to 130 ng/kg/h (controls 52 ng/kg/h) on the day of nalorphine injection. These high rates of output correspond to a mean of 25 μg adrenaline and 12 μg noradrenaline per dog in one day. Assuming a urinary excretion of 2 to 3 per cent of the adrenaline released from the adrenal glands into the blood stream (Euler, 1956, p. 285), the adrenaline output following nalorphine would correspond to 0.8—1.2 mg adrenaline released in each dog during 24 hours. The adrenals of these dogs were later found to contain on the average 1.1 mg/dog. A release of this magnitude would be likely to exhaust the adrenaline content of the adrenal glands unless the resynthesis were to be very rapid.

The 'immediate' response to nalorphine (first four hours after injection) in some morphine tolerant dogs was dominated by adrenaline while the maximal noradrenaline increase appeared later. Analogously with the findings in withdrawal experiments the noradrenaline rise outlasted the adrena-

CHAPTER VII

GENERAL DISCUSSION

The results presented have shown that biologically active monoamines in the brain form characteristic patterns of distribution and release both during long term treatment with morphine and after withdrawal Marked alterations have been found in the catecholamine content of brain adrenals and urine whereas the 5 hydroxytryptamine metabolism remained essentially unaffected

A comparison was performed between two methods for the estimation of catecholamines in brain extracts *i.e.* the method of BERTLER *et al* (1958) and the method of EULER and LISHAJKO (1961) It was found that the extraction fluid (0 4 N perchloric acid) recommended by BERTLER *et al* (1958) for catecholamines and by BERTLER (1961) for 5 hydroxytryptamine was not a suitable medium for the long term storage of the amines A slow gradual reduction of noradrenaline was noticed when extracts were stored for more than a week at $-30°$ C The disappearance of 5 hydroxytryptamine occurred at a much higher rate and was noticeable already a few hours after extraction If however the pH of the extract was adjusted to 3 5—4 0 the catecholamines were stable for several months and 5 hydroxytryptamine for more than three weeks In the method of EULER and LISHAJKO (1961) the addition of the disodium salt of ethylene diaminetetraacetic acid (EDTA) to the brain extracts was omitted, since it was found to reduce the noradrenaline values considerably When these and a few other minor methodological modifications were observed both methods gave essentially the same results with regard to accuracy and specificity for the purpose of the present investigation

The *acute* administration of morphine was associated with a decrease of catecholamines in brain and adrenals (observed in rats and cats) together with an increased urinary output of catecholamine (observed in dogs and rats) These findings confirm earlier reports of a catecholamine releasing action of morphine in acute experiments (*cf* p 11) In rats the injection of 20 or 30 mg/kg of morphine gave a reduction of the adrenal gland content of adrenaline The brain stores of noradrenaline on the other

adrenaline was increased per ml of urine, indicating that the increased output of these amines could not be explained solely as an effect of diuresis 5 HIAA, on the other hand, was decreased when calculated per ml of urine The rise in 5 HIAA might thus have been secondary to the nalorphine induced rise in diuresis

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The *acute* administration of morphine was associated with a decrease of catecholamines in brain and adrenals (observed in rats and cats) together with an increased urinary output of catecholamine (observed in dogs and rats) These findings confirm earlier reports of a catecholamine releasing action of morphine in acute experiments (*cf* p 11) In rats the injection of 20 or 30 mg/kg of morphine gave a reduction of the adrenal gland content of adrenaline The brain stores of noradrenaline on the other

hand, were unaffected by these doses and were reduced only after two consecutive injections of 30 + 30 mg/kg. MAYNERT and KLINGMAN (1962) have recently reported a correspondingly dissociated catecholamine response in brain and adrenals after acute morphine administration in dogs and rabbits, small doses giving a depression of adrenal but not of brain catecholamines. In rats they noticed only a lack of correlation between the magnitude of change in the two organs. These observations indicate either a greater discharge from the adrenals, or a slower rate of resynthesis compared with the brain.

After administration of morphine and other sympathetic stimulating agents in cats VOGT (1954) found a correlation between the power of a drug to cause loss of noradrenaline from the hypothalamus and loss of amines from the innervated adrenal medulla. The present experiments confirmed these observations in cats, since the acute administration of 30 mg/kg of morphine produced a 50—80 % reduction of catecholamines both in brain and adrenals. After five days of morphine treatment a difference between the relative catecholamine content in brain and adrenals became evident also in this species. A replenishment had occurred in the brain noradrenaline, while the adrenal glands were still depleted of adrenaline. However, as evidence of a beginning replenishment of catecholamines in the adrenal glands as well, there was a slight increase of noradrenaline compared with the acute experiment.

The differences noticed between brain and adrenals in acute (rats) and 5 days experiments (cats), thus may be explained by different turn over rates of catecholamines in the two organs.

There have been differences in opinion concerning the rate of resynthesis of catecholamines both in the brain and in the adrenal glands. Some observers (BUTTERWORTH and MANN 1957 a, b; LADE and WOOD 1958) found the turn over rate of the adrenals to be slow, while others have concluded that it is rapid (*cf.* BYGDEMAN, EULER and HÖKFELT 1960). The brain was reported to have a rapid turn over of catecholamines (CARLSSON, LINDQVIST and MAGNUSSON 1959; SPECTOR, MELMON and SJOERDSMA 1962), while BRODIE, SPECTOR and SHORE (1959 b) found it to be relatively slow. BERTLER (1961) concluded that the turn over rate of the rabbit brain is faster than that of the adrenals. Whether differences in turn over rates in brain and adrenals represent the only explanation of the present results, remains an open question.

In *long term* experiments on dogs and rats the catecholamines in brain, adrenals and urine tended to return to control levels.

In rats the noradrenaline depletion of the acute experiment was even converted into an increase of the brain levels The 5 hydroxytryptamine level remained unchanged however indicating that the monoamine oxidase system is intact It was shown that the raised level of noradrenaline was still present in morphine tolerant rats (compared with controls) after administration of drugs interfering with the storage capacity (α methyl DOPA and reserpine) and metabolic break down (the monoamine oxidase inhibitor malamid) These findings seem to suggest that the main difference between controls and morphine tolerant rats is not found in the rate of metabolic break down Thus one probable explanation of the elevated brain level of noradrenaline in morphine tolerant rats may be an increased rate of synthesis

On the other hand the possibility of an increased storage capacity should not be disregarded for the following reasons It was found that malamid produced a state of alertness and excitation in rats after a lag period of a few hours This stage of increased alertness is generally ascribed to an overflow of free catecholamines in the brain tissue (BRODIE *et al* 1959 b) Since the lag time was prolonged in morphine tolerant rats compared with controls this would indicate an increased catecholamine binding capacity rather than an increased rate of synthesis in the brains of morphine tolerant animals Possibly both mechanisms are at work at the same time

In morphine tolerant dogs there was no corresponding increase in the brain content of catecholamines This finding may be a correlate to the behavioral differences between the two species Dogs are sedated while rats are excited from long term morphine administration

The adrenal gland content of adrenaline was normalized during chronic morphine treatment both in dogs and rats in the present study MAYNERT and KLINGMAN (1962) however reported an increase of the adrenal gland content of catecholamines in rats and rabbit during chronic morphine treatment No explanation for this difference in results can be given at present

The urinary catecholamines which were elevated by the first morphine injections showed a decline as a result of tolerance The noradrenaline output gradually became normal and adrenaline was stabilized on a slightly elevated rate of excretion which seemed to be maintained as long as morphine was given These findings suggest that the adaptation to long term morphine administration is associated with a reduced liberation of catecholamines compared with the acute experiments The return to normal of the brain and adrenal tissue levels during chronic morphine administration thus may be explained not only on the basis of a stimulated

resynthesis, but partly as the result of a decreased release of catecholamines

The brain level of 5 hydroxytryptamine in dogs and rats was unaffected by chronic morphine administration Minor fluctuations in the urinary output of 5 hydroxyindoleacetic acid (5-HIAA) might well be explained by changes in the diuresis The findings seemed to indicate that 5 hydroxytryptamine was not involved in the monoamine release, nor were there any signs of an accelerated production of this substance in chronic morphine administration This is in agreement with earlier findings in acute and long term morphine experiments (p 12)

Abrupt withdrawal and *nalorphine induced abstinence* were associated with a rapid mobilization of the catecholamine stores in brain and adrenals There was a reduction of noradrenaline and dopamine in the brain and of adrenaline in the adrenal glands of dogs, together with an increased urinary output of adrenaline and noradrenaline The reduction of adrenaline in the adrenal gland was probably prevented by sectioning of the ipsilateral splanchnic nerve (2 dogs) and could thus be regarded as a central effect, mediated through nervous stimulation

The close relationship between a release of catecholamines in the brain and certain states of excitation with autonomic disturbance has been demonstrated in many earlier investigations (p 9) In those earlier studies the role of the catecholamines in the observed syndrome was sometimes obscure, owing to the presence of various drugs which may have exerted a direct action on the nervous tissue In the present investigation a catecholamine release was noticed in two different experimental situations 1) In acute experiments a release was seen in the non tolerant organism in the *presence* of morphine and 2) following abrupt withdrawal a corresponding release was seen in morphine tolerant animals in the *absence* of the drug The symptom pictures were different in these two instances In the acute experiment there was depression succeeded by excitation in the rats, but only excitation in cats In non tolerant dogs given a large dose (200 mg/kg) of morphine MAYNERT and KLINGMAN (1962) noticed mild convulsions', together with a decrease of catecholamines in brain and adrenals Thus, the symptoms of the acute experiments vary but generally include some signs of excitation In the withdrawal experiments on dogs the symptoms are purely excitatory and the catecholamine pattern in brain adrenals and urine indicates a rapid release These findings support the view that a central liberation of catecholamines is associated with signs of excitation, but that the presence of a drug can obscure the symptom picture

The close connection between the catecholamine release in morphine withdrawal and the symptoms of excitation appears from 1) the agreement between the *degree* of abstinence intensity and the degree of reduction of the catecholamines in the brain, and 2) the coincidence in *time* of the maximal intensity of the abstinence symptoms and the maximal urinary output of catecholamines

In morphine tolerant rats and dogs the urinary output of adrenaline and noradrenaline was maximal on the second and third days of abrupt withdrawal and at this time the adrenaline content of the adrenal glands was reduced In dogs the most prominent excitatory symptoms in withdrawal and nalorphine induced abstinence were connected with the most pronounced depletion in the brain catecholamines (noradrenaline and dopamine) In rats there was no depletion of brain noradrenaline in the present abstinence experiments The symptoms consisted of a combination of drowsiness and irritability together with several autonomic manifestations In earlier experiments a small reduction of brain noradrenaline was noticed in a different rat strain during withdrawal from chronic morphine administration (GUNNE 1959) A moderate noradrenaline release in the brain of morphine tolerant rats need not deplete the brain stores since the release might well be concealed by the rapid resynthesis of brain noradrenaline As mentioned previously the long term morphine experiments in rats (including a final injection of malamid α methyl DOPA or reserpine) have given evidence of an increased rate of synthesis of noradrenaline in the brain

Before definite conclusion can be drawn from changes in the tissue levels of monoamines some information must be obtained concerning the rate of metabolism of the substances studied Such information is furnished by studies of the urinary output which readily reflects changes in the rate of synthesis and liberation (*cf* EULER 1956 p 285) The urinary content of catecholamines reflects those changes more dramatically than the total metabolic end products but of course they give no information concerning the catabolic routes It is true that the monoamines of the brain do not appear in the urine (they do not readily pass the blood brain barrier furthermore they are quantitatively insignificant) However second hand information may be obtained also concerning the neurochemical events in the brain since a stimulation of hypothalamic brain centers which liberates the brain catecholamines is generally reflected by a concomitant liberation from adrenals and peripheral adrenergic nerve terminals (VOGT 1959)

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brain content of γ-aminobutyric acid (GABA) was unchanged in morphine abstinence in the dog. — It has been shown that the stress of morphine withdrawal involves an increased activity also of the adrenal cortex both in man (EISENMAN *et al* 1958, EISENMAN, FRASER and BROOKS 1961) and in rats (PAROLI and MELCHIORRI 1961). In view of the clinical effects of administered corticosteroids it seems improbable, however, that these hormones could explain the symptom pattern in morphine abstinence.

HIMMELSBACH (1943) remarked that the symptoms of morphine abstinence are indicative of a disturbance in the function of the vegetative nervous system, the homeostasis of which was connected with autonomic hypothalamic centers. — Recently the catecholamine and possibly also the 5-hydroxytryptamine stores of the brain stem nuclei were visualized by histochemical fluorescence technique (FALCK 1962, CARLSSON, FALCK and HILLARP 1962). They concluded that adrenergic neurons may exist and noradrenaline may serve as a synaptic transmitter in the central nervous system. Their observations provide direct evidence for a transmitter function of the central monoamines and thus strengthen the hypothesis that the specific alterations in brain noradrenaline content during morphine administration and morphine abstinence reflect biochemical neuronal changes in the regions which control autonomic activity. It also emerges from the present investigation that, in spite of species differences, there are several common features in the neurochemical patterns associated with morphine administration and abstinence in dogs and rats and that minor differences may be correlated to behavioral differences. This implies a probability that the present results will facilitate an understanding of the corresponding mechanisms in human morphine addicts.

levels They concluded that the adaptations on withdrawal of morphine are not stressful in the rat, but are in the dog ' However, the present investigation has shown, mainly by studies of the urinary output, that there are probably no principal species differences between dogs and rats with regard to the catecholamines in morphine abstinence On the contrary a conspicuous *similarity* was found between the patterns of catecholamine output in the two species during withdrawal *from chronic morphine treat* ment

5 Hydroxytryptamine seemed to be unaltered *also* in connection with morphine abstinence The brain levels were unchanged during abstinence irrespective of the intensity of symptoms Changes in the urinary 5 HIAA were probably secondary to the great fluctuations in diuresis connected with withdrawal or nalorphine induced abstinence

The exact role of the catecholamine release in the production of the *morphine withdrawal* syndrome is difficult to decide at present Many stressful agents cause *an increased activity in the sympathetic nervous* system and increase the secretion from the adrenal glands It should be recalled *however, that the drug induced* depletion of hypothalamic nor adrenaline, first reported by VOGT (1954) was not elicited by all the drugs tested, in spite of the use of sublethal doses Only when more or less con spicuous symptoms of autonomic activity were evoked, a depletion of brain noradrenaline was found Thus the drugs active on hypothalamic cate cholamines had the common feature of stimulating sympathetic activity VOGT (1957) showed that nalorphine counteracted the behavioral as well as the noradrenaline depleting effects of morphine in the cat brain Again the effect on catecholamines was associated with the autonomic and be *havioral response* MAYNERT and KLINGMAN (1962) were able to prevent the noradrenaline depletion of nalorphine in the brain of morphine tolerant dogs, when pentobarbital anaesthesia was given, *in order to abolish the* abstinence excitation

Together with the present results these findings suggest that the release of brain catecholamines in morphine abstinence is secondary to a vigo rous stimulation of sympathetic brain centers However *the concept* of a nervously mediated stimulation as the cause of the catecholamine *depletion* does not contradict the view that a liberation of these substances *in the* brain stem, might have a decisive influence on the symptom *pattern in* morphine abstinence

The possible role of other biologically active substances in the *brain must* be clarified before the relation between brain catecholamines and *morphine* effects can be finally evaluated MAYNERT *et al* *(1962) reported that the*

stem and cerebellum) The magnitude of the reduction was related to the severity of the abstinence symptoms In the brain stem there was a 21, 41 and 59 per cent reduction in abstinence in grades I II and III respectively A concomitant reduction of dopamine was seen in dogs with moderate and marked (grade II and III) abstinence symptoms but did not appear when the symptoms were slight (grade I) The 5 hydroxytryptamine of the brain stem was unaltered at any degree of abstinence — In rats there were no changes of brain noradrenaline or 5 hydroxytryptamine during morphine abstinence

4 *Adrenals* *Acute* administration of morphine reduced the adrenaline and noradrenaline content of the adrenal glands in rats and cats After five days of morphine administration in cats there was still a reduction of both amines but a beginning replenishment of noradrenaline was noticed After *chronic* morphine administration in dogs and rats a normal content of adrenaline and noradrenaline was found in the adrenal glands

Following *withdrawal* of chronic morphine administration there was a reduction of adrenaline in the adrenal glands of dogs and rats *Nalorphine induced abstinence* reduced the adrenaline level in the adrenals of dogs There was a 49 52 and 68 per cent reduction in abstinence in grades I, II and III respectively The adrenaline depletion was probably mediated *via* the splanchnic nerves ince after unilateral splanchnic sectioning only the innervated gland contained definitely subnormal amounts of adrenaline (two dogs) — In morphine tolerant rats nalorphine did not influence the catecholamine content of the adrenals

5 *Urine* The *first* few morphine injections caused an increase of the urinary output of adrenaline and noradrenaline in dogs and *rats After repeated* injections there was a return of the two catecholamines towards the normal level The noradrenaline output returned to normal while adrenaline remained supranormal in both species as long as morphine was given The output of 5 HIAA during long term morphine administration appeared only to reflect changes in the diuresis both *in dogs and rats*

Following *abrupt withdrawal* there was a marked rise of urinary adrena line and noradrenaline in dogs and rats The *increase* of noradrenaline outlasted the increase of adrenaline by 2 to 3 days in both *species* A cor responding pattern of catecholamine excretion was also noticed in dogs when abstinence was produced by *nalorphine* — The urinary 5 HIAA rose in morphine tolerant dogs on the first day of withdrawal or adminis tration of nalorphine but a decrease was seen in rats Again the fluctuations of urinary 5 HIAA might have been secondary to changes *in diuresis* since

SUMMARY

1 The effects of long term administration of morphine and of abrupt withdrawal as well as of nalorphine induced abstinence have been studied, mainly with reference to the brain content of noradrenaline, dopamine and 5 hydroxytryptamine (serotonin), the adrenal gland content of adrenaline and noradrenaline and the urinary output of adrenaline, noradrenaline and 5 hydroxyindoleacetic acid (5 HIAA) The study was carried out on dogs, rats and cats The brain noradrenaline was determined either according to BERTLER, CARLSSON and ROSENGREN (1958) or EULER and LISHAJKO (1961) These methods were compared and a few modifications were suggested

2 The general appearance and behavior of the animals were noticed during acute and long term administration of morphine and in abstinence produced by nalorphine or by abrupt withdrawal The abstinence syndrome in rats was found to contain some apparently inconsistent elements signs of excitation (irritability and disturbed autonomic functions), together with drowsiness and eyelid ptosis The abstinence syndrome in dogs however, was purely excitatory A simple rating scale for abstinence symptoms in dogs was devised The symptoms were scored as slight (grade I), moderate (grade II) or marked (grade III)

3 *Brain Acute* administration of morphine reduced the brain noradrenaline in rats and cats After five daily morphine injections the brain noradrenaline was restored in cats Following *chronic* morphine treatment the brain level of noradrenaline, dopamine and 5 hydroxytryptamine was normal in dogs In rats the noradrenaline level was supranormal after chronic morphine treatment, while the brain content of 5 hydroxytryptamine was unaltered A monoamine oxidase inhibitor (nialamid) caused a greater increase of noradrenaline in morphine tolerant rats than in controls The noradrenaline releasing agents *α* methyl DOPA and reserpine produced a smaller noradrenaline decrease in brains of morphine tolerant rats than in controls

During *withdrawal* and *nalorphine induced abstinence* there was in dogs a reduction of noradrenaline in all parts of the brain (telencephalon, brain

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corresponding alterations were noticed in the urine volumes of the two species

6 The following *general conclusions* are drawn from the present findings

a) Acute administration of morphine produces an activation of sympathetic parts of the nervous system, central as well as peripheral, eventually leading to a depletion of brain and adrenal stores of catecholamines in rats and cats Long term administration of morphine induces an increased rate of resynthesis of catecholamines, probably as a response to the increased demands by the stimulated tissue Signs of an accelerated resynthesis are found in brain and adrenals of rats and cats and in the adrenals of dogs during chronic morphine treatment

b) Withdrawal and nalorphine induced abstinence elicits a rapid liberation of brain and adrenal catecholamines and the *intensity* of the abstinence syndrome in dogs is proportional to the depletion of brain catecholamines Furthermore the maximal symptoms coincide in *time* with the maximal urinary output of catecholamines There are species differences between dogs and rats with regard to the behavioral manifestations of abstinence These are reflected by quantitative differences in the catecholamine depletion between the two species The general patterns of catecholamine output following morphine withdrawal are, however, essentially the same in dogs and rats

These findings seem to link the appearance and development of the morphine abstinence syndrome with a liberation of brain catecholamines (noradrenaline and dopamine) 5 Hydroxytryptamine appears to remain unaffected by chronic morphine treatment as well as by withdrawal

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CONTENTS

I PREFACE

The present investigation is part of a research project dealing with the influences of various stress factors on the pulmonary gas exchange and on the control of respiration The experimental part of the investigation was carried out in 1961

It is a pleasure to record my gratitude to Professor U S v EULER Head of the Department of Physiology I who originally introduced me to the physiology of respiration and evoked my interest in this field of research I also wish to convey my sincere thanks to Drs H BJURSTEDT and C M HESSER who have been my teachers since I joined their research groups in 1958 and who have generously placed the facilities of their laboratories at my disposal

Finally I would like to express my heartfelt thanks to members of the laboratory staff whose never failing interest and collaboration made the experiments possible and to J BRISMAR M K who assisted in the processing of the experimental data

Stockholm April 1963

GEORG MATELL

II INTRODUCTION

Seventy five years have passed since GEPPERT and ZUNTZ in *1888 published* their classical studies on the mechanisms involved in the hyperpnea of exercise. The wealth of literature available *to day on this engrossing subject has un*doubtedly increased our knowledge of the numerous factors and influences which *have to be considered. In spite of the efforts devoted to elucidating* the nature of exercise hyperpnea, and the greatly increased interest prompted *during recent years by the development of new tools for its study, many basic* questions pertinent to the basic problem still remain unsettled.

The present investigation is a study of certain dynamic characteristics of ventilatory and blood chemical changes in man resulting from moderate, *constant load, dynamic exercise. On the whole, information is lacking as to* the interrelations between the time courses of ventilation and arterial O_2 and CO_2 *tensions during constant load exercise and subsequent recovery. In the* present work continuous and simultaneous recordings of *i. e.* pulmonary ventilation and arterial O_2 saturation and pH have been used to permit a study of the above mentioned interrelationships and of the interdependence of ventilation and blood chemical changes. The study has been devoted especially to the changes at the transitions from rest to work and from work to rest, since such changes were expected to be more informative than those observed in the steady state of ventilation.

In order to judge, if possible, the relative importance of the H^+ CO_2 complex, and of its components, in eliciting and maintaining exercise hyperpnea, the changes resulting from a given work load were also compared with those observed during CO_2 inhalation and after the bicarbonate content of blood had been artificially changed by ingestion of ammonium chloride and sodium bicarbonate respectively.

List of Symbols and Abbreviations

Primary Symbols

V Gas volume in general
$\dot{V}$ Gas volume per unit time
P Pressure in general including partial pressure
S Oxygen saturation of hemoglobin per cent
F Fractional concentration in dry gas phase

Suffixes

(a) Gas phase

I Inspired gas
A Alveolar gas
E Expired gas
T Tidal gas

(b) Blood phase

a Arterial
v Venous

The above symbols are those of the Atlantic City Convention (PAPPENHEIMER *et al* 1950)

Some Other Symbols

STPD	Standard temperature and pressure dry (0 C, 760 mm Hg)
BTPS	Body temperature and pressure saturated with water vapor
$BHCO_{3S}$	The plasma bicarbonate content (mM/l) of blood under standard conditions *i e* at 37 pH 7 4 and saturated with oxygen
NB	Normal $BHCO_{3S}$
HB	High $BHCO_{3S}$
LB	Low $BHCO_{3S}$
BSA	Body surface area m^2

Other symbols are defined in the text as they occur

Gas volumes are expressed in liters at the true volumes in the body (BTPS) except $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ which are expressed in l/min STPD $\dot{V}_T$ is expressed in liters BTPS/min/m^2 BSA except when otherwise stated Gas tensions are given in mm Hg at actual body temperature

1 kpm = normal gravitational force on 1 kg
1 kpm/min = 0 163 W = 7 32 ft lb/min

II INTRODUCTION

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The present investigation is a study of certain dynamic characteristics of ventilatory and blood chemical changes in man resulting from moderate, constant load, dynamic exercise On the whole, information is lacking as to the interrelations between the time courses of ventilation and arterial O_2 and CO_2 tensions during constant load exercise and subsequent recovery In the present work continuous and simultaneous recordings of i a pulmonary ventilation and arterial O_2 saturation and pH have been used to permit a study of the above mentioned interrelationships and of the interdependence of ventilation and blood chemical changes The study has been devoted especially to the changes at the transitions from rest to work and from work to rest, since such changes were expected to be more informative than those observed in the steady state of ventilation

In order to judge, if possible, the relative importance of the H^+ CO_2 complex, and of its components, in eliciting and maintaining exercise hyperpnea, the changes resulting from a given work load were also compared with those observed during CO_2 inhalation and after the bicarbonate content of blood had been artificially changed by ingestion of ammonium chloride and sodium bicarbonate, respectively

BARR, HIMWICH and GREEN demonstrated an increased acidity of the arterial blood during exercise. In recent years, with the advent of refined measuring techniques for blood pH, several investigators have confirmed the occurrence of acidemia during exercise; spot sampling techniques were used almost exclusively. The bulk of evidence is in favour of a slight shift towards acidosis during moderate exercise (GALDSTON and WOLLACK 1947, HICKAM *et al.* 1951, FILLEY, GREGOIRE and WRIGHT 1954, BARTELS *et al.* 1955, ASMUSSEN and NIELSEN 1958, BARR *et al.* 1963), the shift being more marked during heavy and exhausting work (MITCHELL, SPROULE and CHAPMAN 1958, HOLMGREN and LINDERHOLM 1958, LAMBERTSEN *et al.* 1959).

Opinions also differ with respect to the effects on the alveolar P_{O_2}. An increase during submaximal work has been reported by LILIENTHAL *et al.* (1946), FILLEY *et al.* (1954), BARTELS *et al.* (1955), whereas SUSKIND *et al.* (1950) observed a fall in the alveolar P_{O_2}. Direct determinations of the arterial P_{O_2} have generally shown rather small changes during exercise, although there is less agreement as to the direction of the changes. Thus, a rise was observed by BARTELS *et al.* (1955) and LAMBERTSEN *et al.* (1959), while LILIENTHAL *et al.* (1946), SUSKIND *et al.* (1950), FILLEY *et al.* (1954), HOLMGREN and LINDERHOLM (1958) have reported a fall in the arterial P_{O_2}. ASMUSSEN and NIELSEN (1958) and MITCHELL *et al.* (1958) found no significant change in the arterial P_{O_2} during exercise. For exhausting exercise HOLMGREN and LINDERHOLM (1958) reported an impressive fall in the arterial P_{O_2} (22 mm Hg).

There are as yet relatively few investigations in which alveolar or arterial P_{CO_2} and P_{O_2}, or arterial pH, have been followed by means of consecutive or continuous measurements during the course of exercise. RAHN and OTIS (1949), who plotted the alveolar tensions as a function of time on the CO_2-O_2 diagram, observed an initial lowering of the alveolar P_{O_2} and an increase in the alveolar P_{CO_2}. SUSKIND *et al.* (1950) described a similar initial course for the arterial gas tensions. BARR *et al.* (1963) studied the time courses of blood gas changes during moderate, constant load exercise in man, using techniques for continuous and simultaneous recordings of arterial O_2 saturation and pH. Quantitative ventilatory measurements were avoided in order not to interfere with the normal respiratory pattern. After an initial drop during the first 1.5 min of exercise the calculated arterial P_{O_2} increased by about 5 mm Hg to reach a plateau in the 3rd—6th min of exercise. The arterial pH was lowered by about 0.04 units. It was noted that the arterial gas tensions and pH remained unchanged during the first half minute of exercise and recovery.

Interaction of Humoral and Non-Humoral Stimuli in the Hyperpnea of Exercise

Ventilatory responses to the H^+-CO_2 complex. It is a well known fact that in resting man the ventilatory response to changes in the arterial $[H^+]$ and P_{CO_2}, as produced by inhalation of CO_2, is characteristically linear over a certain range of arterial $[H^+]$ or P_{CO_2} values (*cf.* LAMBERTSEN *et al.* 1953, SCHAEFER

III BACKGROUND

As an introduction to the problems dealt with in the present work, a short survey of certain earlier investigations will first be given on changes in arterial blood gas levels during exercise Subsequently, other investigations will be reviewed which pertain to the interaction of humoral and non humoral stimuli involved in exercise hyperpnea The text is not intended to supply a detailed account of the numerous investigations dealing with these topics For the sake of clarity only such contributions are included which were considered essential for the framing of the problems

The current interest in topics dealing with the mechanisms underlying the hyperpnea of exercise is reflected in several excellent reviews which have appeared during the last few years For a more detailed analysis of the various aspects of the hyperpnea of exercise the reader may consult the recent reviews presented by DEJOURS (1959 and 1962), and by LAMBERTSEN (1961) and by several authors in The Regulation of Human Respiration' (Proceedings of the J S Haldane Centenary Meeting in Oxford 1961) and in the British Medical Bulletin (January 1963)

Effects of Exercise on Arterial Blood Gases and Acid Base Balance

Numerous attempts have been made to evaluate the roles of the known blood borne respiratory stimuli in the initiation and maintenance of exercise hyperpnea The majority of these investigations have been focussed on changes in the arterial blood, as judged either by analysis of alveolar gas or by direct determination of blood gases and acid base balance following spot sampling' of arterial blood

A perusal of the results obtained by use of the above mentioned methods makes it evident that there is a lack of agreement as to the magnitude and direction of deviations in alveolar or arterial gas tensions resulting from exercise Thus, in submaximal work several investigators have found an increase in the alveolar P_{CO} (HALDANE and PRIESTLEY 1905, DOUGLAS and HALDANE 1912, ASMUSSEN and NIELSEN 1946, SUSKIND *et al* 1950, BANNISTER *et al* 1954) whereas others have observed a fall (*cf* COMROE 1944) A rise in the arterial P_{CO} was observed by SUSKIND *et al* (1950) and HICKAM *et al* (1951) BARTELS *et al* (1955) reported a fall in the arterial P_{CO}, whereas other authors have found only slight fluctuations within the normal range of variation (LILIENTHAL *et al* 1946 FILLEY *et al* 1954 ASMUSSEN and NIELSEN 1958 HOLMGREN and LINDERHOLM 1958)

Direct determinations of the arterial pH during exercise have yielded more consistent results Already in 1923 ARBORELIUS and LILJESTRAND as well as

BARR HIMWICH and GREEN demonstrated an increased acidity of the arterial blood during exercise In recent years with the advent of refined measuring techniques for blood pH several investigators have confirmed the occurrence of acidemia during exercise spot sampling techniques were used almost exclusively The bulk of evidence is in favour of a slight shift towards acidosis during moderate exercise (GALDSTON and WOLLACK 1947 HICKAM *et al* 1951, FILLEY GREGOIRE and WRIGHT 1954 BARTELS *et al* 1955 ASMUSSEN and NIELSEN 1958 BARR *et al* 1963) the shift being more marked during heavy and exhausting work (MITCHELL SPROULE and CHAPMAN 1958 HOLMGREN and LINDERHOLM 1958 LAMBERTSEN *et al* 1959)

Opinions also differ with respect to the effects on the alveolar P_O An increase during submaximal work has been reported by LILIENTHAL *et al* (1946) FILLEY *et al* (1954) BARTELS *et al* (1955) whereas SUSKIND *et al* (1950) observed a fall in the alveolar P_O Direct determinations of the arterial P_O have generally shown rather small changes during exercise although there is less agreement as to the direction of the changes Thus a rise was observed by BARTELS *et al* (1955) and LAMBERTSEN *et al* (1959) while LILIENTHAL *et al* (1946) SUSKIND *et al* (1950) FILLEY *et al* (1954) HOLMGREN and LINDERHOLM (1958) have reported a fall in the arterial P_O ASMUSSEN and NIELSEN (1958) and MITCHELL *et al* (1958) found no significant change in the arterial P_O during exercise For exhausting exercise HOLMGREN and LINDERHOLM (1958) reported an impressive fall in the arterial P_O (22 mm Hg)

There are as yet relatively few investigations in which alveolar or arterial P_{CO} and P_O or arterial pH have been followed by means of consecutive or continuous measurements during the course of exercise RAHN and OTIS (1949) who plotted the alveolar tensions as a function of time on the CO_2 O_2 diagram observed an initial lowering of the alveolar P_O and an increase in the alveolar P_{CO} SUSKIND *et al* (1950) described a similar initial course for the arterial gas tensions BARR *et al* (1963) studied the time courses of blood gas changes during moderate constant load exercise in man using techniques for continuous and simultaneous recordings of arterial O_2 saturation and pH Quantitative ventilatory measurements were avoided in order not to interfere with the normal respiratory pattern After an initial drop during the first 1 5 min of exercise the calculated arterial P_O increased by about 5 mm Hg to reach a plateau in the 3rd—6th min of exercise The arterial pH was lowered by about 0 04 units It was noted that the arterial gas tensions and pH remained unchanged during the first half minute of exercise and recovery

Interaction of Humoral and Non Humoral Stimuli in the Hyperpnea of Exercise

Ventilatory responses to the H CO₂ complex It is a well known fact that in resting man the ventilatory response to changes in the arterial [H⁺] and P_{CO} as produced by inhalation of CO_2 is characteristically linear over a certain range of arterial [H] or P_{CO} values (*cf* LAMBERTSEN *et al* 1953 SCHAEFER

1958) The slope of the CO_2 response curve, as well as its transposition to the left or right in acid base displacements have been studied extensively by several investigators (NIELSEN 1936b, NIELSEN and SMITH 1951, ALEXANDER *et al* 1955, LERCHE *et al* 1959, CUNNINGHAM *et al* 1961) That the resting CO_2 response curve becomes steeper under the influence of hypoxia has been shown by NIELSEN and SMITH (1951) and by many others (*cf* LLOYD 1963) although KELLOG *et al* (1957) found the slope to be unchanged in acclimatized subjects

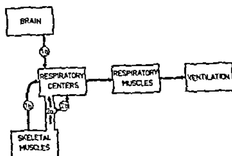
The ventilatory response to CO_2 inhalation has also been investigated during exercise and found to be the same as at rest During exercise, however, the rectilinear part of the stimulus response curve is displaced to the left of the resting curve (NIELSEN 1936b, CRAIG 1955, NIELSEN and ASMUSSEN 1963), the more so the higher the work intensity (ASMUSSEN and NIELSEN 1957) NIELSEN and ASMUSSEN (1963) have demonstrated evidence for the existence of a CO_2 threshold during exercise, which must be surpassed before ventilation is stimulated by CO_2

Information is scanty as to the effects of metabolic acid base displacements on the ventilatory response to exercise HALDANE in 1921 noted severe panting during exercise under ammonium chloride acidosis DENNIG *et al* (1930) observed, in one subject, an increased ventilatory response to exercise after ingestion of ammonium chloride, and conversely, a slightly diminished ventilatory response during alkalosis REFSUM (1961) observed, also in one subject, an enormous ventilation in one minute exercise tests after ingestion of ammonium chloride In the mentioned investigations the magnitude of the acid base displacements was considerable however

It is of special interest to consider what the ventilation of a resting man would be if his blood had the same acid base composition as would occur during exercise It would then be possible to quantitatively express the non humoral drive in exercise hyperpnea by subtracting the chemically accountable ventilation from that actually measured during exercise This approach has been used by CUNNINGHAM (1963) Such calculations showed that the average changes reported to occur in the H^+ CO_2 complex in exercise of various severities (assuming the arterial oxygen tension to be the same for rest and exercise) could be expected to yield in the resting subject, a ventilation that would amount to about 60 % of the exercise ventilation It was pointed out that this estimate was based on certain assumptions as to the slope of the CO_2 response curve and that the effect of raised body temperature was not included in the calculations

Some features of the combined neuro humoral concept The older notions that a single, dominant mechanism was responsible for the hyperpnea of exercise *e g* chemical (HALDANE and PRIESTLEY 1905 WINTERSTEIN 1911 DOUGLAS 1914) or neurogenic (reflex or cortical) have gradually been abandoned in favour of the more recent concept of a multiplicity of regulating factors (for

Fig 1 Schematic representation of factors and pathways involved in the mechanism of exercise hyperpnea (modified from DEJOURS 1959) Overriding control functions (1a) interact with reflex influence originating in the muscles (1b) and with blood borne stimuli (chemical as e g [H] CO_2, O_2, catecholamines and physical as e g body temperature and blood pressure) which either act directly on the respiratory centers (2a) or via peripheral chemo- or baroreflexes (2b)



reviews see COMROE 1944 GRAY 1950 GRODINS 1950 WINTERSTEIN 1955 SCHMIDT 1956 DEJOURS 1959 LAMBERTSEN 1961 CUNNINGHAM 1963)

Fig 1 shows postulated nervous pathways along which (1a) overriding control is exerted from higher levels in the brain and (1b) reflex influence from musculoskeletal receptors are mediated Also represented are the pathways through which blood chemical changes caused by the production of metabolites in the working muscles may influence the activity of the centers These chemical changes (blood borne stimuli) may exert their action directly via the blood perfusing the centers (2a) or by reflex influence (2b) originating in peripheral chemo- or baroreceptors

That the hyperpnea of exercise cannot be fully explained in terms of the well known stimuli to breathing (CO_2 blood and tissue acidity and hypoxia) or other known chemical changes related to metabolism has thus long been recognized by different groups of investigators Certain interrelations between other factors capable of modifying ventilation and some characteristics of exercise hyperpnea are illustrative in this respect

Thus by using the relatively simple approach of studying the time course of the ventilatory response to exercise it has been possible to draw certain conclusions as to the import of factors other than chemical This approach was probably first used by KROGH and LINDHARD (1913b) who observed that ventilation rises very quickly at the onset of exercise They concluded that the initial ventilatory change occurred too rapidly to result from any action of blood borne stimuli on the respiratory center

The observations of KROGH and LINDHARD have in the main been confirmed by more recent investigations Thus a sudden onset of light dynamic exercise has been shown to result in an immediate increase in ventilation (ASMUSSEN and NIELSEN 1948 DEJOURS *et al* 1955a) In its further time course ventilation first remains relatively constant during the 20—30 seconds following the onset of exercise and then shows a secondary increase to a plateau (DEJOURS 1963) With a sudden transition from exercise to rest ventilation again falls abruptly (ASMUSSEN and NIELSEN 1948 DEJOURS *et al* 1955a) and then

remains essentially constant at a higher than pre exercise level for 20—30 seconds before returning gradually to normal The sequence of ventilatory changes is characteristic for periods of light to moderate dynamic exercise under *constant load*

It is the consensus of opinion that both the initial rise and terminal fall of exercise hyperpnea must be neurogenic in nature (fast component' of DEJOURS), and that the neurogenic control of ventilation in these phases is at least partially exerted via proprioceptive reflexes from muscles and tendons in the moving limbs (HARRISON, CALHOUN and HARRISON 1932, KAO, SCHLIG and BROOKS 1955, HUTT, HORWATH and SPURR 1958, DEJOURS 1959, DEJOURS, BECHTEL LABROUSSE and RAYNAUD 1961) The rapid ventilatory changes at the start and cessation of exercise have been further investigated on a quantitative basis (for reviews, see DEJOURS 1959 and 1963) In general, the fast component has been found to increase somewhat with increasing work loads and to be greater at the cessation of exercise than at its start

The slower phases of the changes in ventilation during exercise and recovery have been connected with both cortical control and with reflex and blood borne influences from the working muscles (for reviews, see DEJOURS 1963, KAO 1963, NIELSEN and ASMUSSEN 1963)

Studies of the slow phase of recovery may be rewarding in attempts to distinguish between humoral and non humoral stimuli By 'autotransfusion of work blood ' ASMUSSEN and NIELSEN (1950) obtained evidence that the blood during heavy exercise contained specific substances with a stimulatory effect on ventilation

In similar experiments DEJOURS MITHOEFER and TEILLAC (1955 b) trapped blood in the legs by inflation of cuffs at the end of light exercise and observed, on release of the cuff pressure during recovery that hyperpnea occurred sharply after a long delay of 18 seconds *i e* not before the blood liberated from the occluded limbs had reached the arterial side Conversely if cuffs around the thighs were inflated just before exercise (V_{O_2} about 0 4 l/min) was stopped, ventilation and alveolar P_{CO} fell below resting levels, indicating withdrawal of the normal stimulation to respiration in recovery (DEJOURS MITHOEFER and RAYNAUD 1957b) A final assessment of the relative importance of humoral and non humoral stimuli during exercise and recovery will have to await experimental analysis of the time course of ventilatory and blood chemical changes, as pointed out by TEILLAC and LEFRANÇOIS (1962) With certain assumptions previously mentioned (p 10) CUNNINGHAM (1963) estimates that hypothetical resting men whose bloods have the same compositions as those of the exercising subjects would in most cases have approximately the same ventilations as would be expected a few seconds after the end of exercise, immediately after the completion of the rapid phase of recovery but before change in the composition of the blood in the receptor areas could occur

IV PROBLEMS

From the investigations mentioned in the preceding review it is evident that the long standing uncertainty as to the changes in arterial P_{CO_2} pH and P_{O_2} occurring during exercise has been a major source of earlier disagreements as to the possible interdependence of ventilatory and blood chemical responses to exercise This uncertainty has made it difficult to appraise quantitatively the various neurogenic and chemical factors which may be responsible for e g the components postulated by the neuro humoral theory

1 Recent investigations performed in this laboratory on human subjects by use of continuous recording techniques have shown relatively consistent changes in the arterial blood gases and pH during constant load moderate exercise and ensuing recovery it became evident that (a) some of the earlier discrepancies may be explained by the observation that the normal oscillations in arterial gas tension levels at rest may be sufficiently great to necessitate caution in drawing conclusions based on results from spot sampling of arterial blood and (b) the arterial pH and O_2 saturation displayed characteristic time courses during the periods of exercise and recovery

It therefore seemed worthwhile to reinvestigate the interdependence of ventilatory and arterial chemical changes in the different phases of constant load moderate exercise using continuous recording techniques for an analysis of the relations between these changes

2 The quantitative appraisal of possible chemical and neurogenic stimuli in exercise hyperpnea has also suffered from the lack of consistent data as to the true changes in arterial blood gases and acid base balance In addition the possible participation of blood borne H^+ and CO_2 in the initiation and maintenance of exercise hyperpnea has been obscured by the scarcity of data relating metabolic ventilatory and blood chemical alterations in a single group of subjects

Therefore an attempt was made to assess quantitatively the possible stimulating effect of the H CO_2 complex in experiments in which the responses of ventilation and arterial $[H^+]$ and P_{CO_2} to constant load moderate exercise were compared with those observed during inhalation of CO_2 at rest The study was extended to include an assessment of the H^+ and CO_2 stimuli in both types of hyperpnea after independent changes in their arterial levels by ingestion of ammonium chloride and sodium bicarbonate

Although the responses to exercise were studied in conditions of normal high and low $BHCO_3$ it should be stressed that the work was standardized throughout the investigation to 625 kpm/min for 6 min on the bicycle ergometer The results obtained from the exercise experiments and the discussion of their possible physiological significance can accordingly not be generalized to hold for other work loads or for other types of work

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V METHODS

The basic feature of the experimental approach was the continuous and simultaneous recording of tidal air and respiratory minute volume as well as arterial O saturation and pH during constant load, dynamic exercise and recovery The ventilatory changes and the simultaneous blood chemical changes were compared with those obtained during CO_2 inhalation In order to study the effects of changing the buffering capacity of the blood and tissues, the experiments were performed both when buffering capacity was normal ('*NB*') and after subchronic elevation and lowering of the $BHCO_{3st}$ were artificially induced These conditions will subsequently be termed *HB* (*high* $BHCO_{3st}$) and *LB*' (*low* $BHCO_{3st}$) *The acid base displacements* were induced by ingestion of ammonium chloride and sodium bicarbonate (see p 18) for three days prior to the experiments

It was decided to use a moderate work load for several reasons First, with *moderate respiratory minute volumes it is possible to keep the breathing* resistance of the measuring devices sufficiently low, so that the respiration is not significantly influenced Second, it seemed desirable to avoid the increasing work of breathing that would accompany an otherwise constant load of heavy exercise (*cf* LILJESTRAND 1918, NIELSEN 1936a, MCKERROW and OTIS 1956 MARGARIA *et al* 1960, MARSHALL 1962) Third, it was considered appropriate to avoid accumulation of large amounts of fixed acids in order to maintain as far as possible, the initial acid base condition

The technique of recording continuously the arterial pH and O_2 saturation involved a loss of blood of about 8 ml per min, *i e 250 ml for the completion* of one experiment According to GULLBRING *et al* (1960) this loss would correspond to a decrease in the physical working capacity at pulse rate 170 (PWC_{170}) of about 75 *kpm/min No adverse effects of blood loss of this order of* magnitude were observed on heart rate and blood pressure in an earlier investigation, in which the same recording techniques were used during exercise at 400 and 700 kpm/min (BARR *et al* 1963)

The *ventilatory response to* CO_2 inhalation is linear over a limited range of CO_2 fractions (for reviews see SCHAEFER 1958 LAMBERTSEN 1961) In the present experiments the CO_2 ventilatory *response curves were investigated* within this range Two different CO_2 fractions were used for this purpose the higher fraction being chosen so as to yield approximately the same $\dot{V}_I$ in the 9th and *10th minutes* as was obtained in exercise during the steady state of ventilation

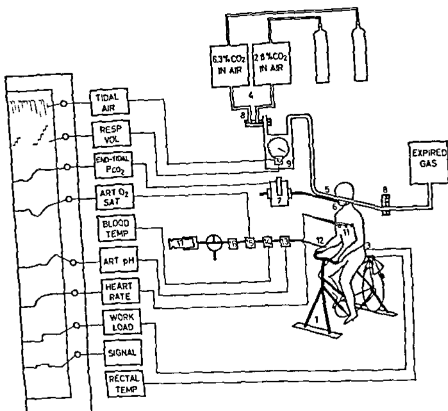


Fig 2 Schematic diagram of exper mental set up 1 = b cycle ergometer 2 = braking load 3 = thermistor for rectal temperature 4 = plastic bags for gas mixtures 5 = respiratory lves and mouth piece 6 = end tidal gas sampler 7 = CO analyzer pick up 8 = three way stopcock 9 – gas meter 10 = two-channel photoelectric pulse adding device 11 = electrodes for card otachometer 12 = rad 1 artery catheter 13 = pH sensing unit 14 = thermistor 15 = oximeter cuvette 16 = roller pump and 17 = syringe for sampling of arterial blood

Techniques

The general experimental arrangement is shown in Fig 2 The variables listed below were inscribed continuously and simultaneously on photokymographic records at a paper speed of 1 25 mm/sec.

Inspired gas volumes were measured with a standard dry rotary gas meter (Nordgas) equipped with a fast electronic pulse-counting device (BJURSTEDT and LOWN 1960) to permit continuous and accurate recording of *tidal volume* and *respiratory minute volume* (staircase curves)

End tidal P_{CO_2} End tidal gas was sampled automatically breath by breath by means of a device described by BRISMAR HESSER and MATELL (1962) and drawn through a rapid infrared CO_2 analyzer (Beckman Spinco model I B-1) at a constant flow of 100 cc/min The end tidal sampler was mounted on a low resistance and low dead space breathing valve modified according to v DOBELN (1949) The *total external*

dead space amounted to 15 ml The *over all external breathing resistance* to a flow of 1 l/sec was 2 6 cm H_2O during inspiration and 0 9 cm H_2O during expiration The CO_2 analyzer was calibrated with five known mixtures of CO_2 in air, saturated with water vapor

pH and O_2 saturation of the arterial blood were recorded by a technique described in detail elsewhere (BARR and BJURSTEDT 1963) Arterial blood was drawn via a Teflon catheter inserted into the radial artery (BARR 1961) through the pH oximeter assembly The flow of blood was kept constant at a rate of 8 ml/min by means of a roller pump

The dynamic response of the recording system was adjusted so that a square wave change in arterial O_2 saturation and pH at the catheter tip in the radial artery resulted in 90 per cent galvanometer deflection after 6 8 sec for both parameters

By using a logarithmic amplifier (WIEDERHIELM 1956) a linear response to changes in arterial O_2 saturation was obtained For each experiment three points on the calibration curve were obtained by equilibrating blood samples from the subject with known gas mixtures at 37° C and by passing these samples through the pH-oximeter sensing units The galvanometer deflections were recorded and the levels of O_2 saturation calculated from the line charts of SEVERINGHAUS (1958) The drift of the oximeter assembly was virtually nil over the experimental periods The technique permitted detection of changes in arterial O_2 saturation as small as 0 1 %

Calibration of the pH electrode assembly was performed before and after each experiment using five 1/15 molar phosphate buffers (pH range 7 00—7 60) (the same buffers were used for all the experiments) prepared according to HASTINGS and SENDROY (1924) The drift of the pH electrode assembly was less than 0 001 pH during the experimental period

The temperature of the blood passing the pH electrode assembly was continuously recorded on an ink writer to permit correction of the arterial pH to actual body temperature using a correction factor of 0 0147 per degree C (ROSENTHAL 1948)

Heart rate was obtained by means of an instantaneous cardiotachometer (STURM and WOOD 1947)

Rectal temperature was followed by use of a thermistor unit

Laboratory methods

Expired air was collected in plastic bags Samples of expired air were withdrawn and stored under mercury for later analysis in duplicate according to SCHOLANDER (1947)

O_2 and CO_2 contents of arterial blood having just passed the pH oximeter assembly were measured in duplicate according to VAN SLYKE and NEILL (1924) The maximal difference between duplicates was 0 2 vol % Corrections were made for syringe dead space

Calculations

For calculation of changes in the arterial O_2 and CO_2 tensions the recorded intra individual changes in arterial pH and S_{O_2} were first reduced to time averages over half minute periods (first 3 minutes of exercise and first 2 minutes of recovery) and one minute periods (3rd—6th minute of exercise and 2nd—6th minute of recovery) The corresponding arterial O_2 and CO_2 tensions were then calculated as follows

Arterial O_2 tension (P_{O_2}) (at body temperature) was computed from the values for arterial oxygen saturation, pH, and body temperature using the line charts of SEVERINGHAUS (1958)

Table I

Subject	Age (years)	Height (cm)	Weight (kg)	Body surface area (m^2)	Blood pressure (mm Hg)	
					Supine	Standing
R. H	22	184	65	1 86	140/90	140/90
J B	22	178	75	1.93	130/80	130/80
A F	21	174	68	1 82	130/80	130/80
E. T	22	182	67	1.87	125/80	125/80
B F	25	175	61	1 74	105/70	100/70
P S	26	190	76	2 02	105/70	105/70
J F	23	181	64	1 82	130/90	125/85
M	23	181	68	1 87	124/80	122/79

From nomogram of DUBOIS and DUBOIS (1916)

Arterial CO_2 tension (P_{CO_2}) (at body temperature) was computed by means of the HENDERSON HASSELBALCH equation from values of pH_{37} plasma CO_2-content and serum pK_{37} for carbonic acid (obtained according to SEVERINGHAUS STUPFEL and BRADLEY 1956) The solubility coefficient for CO_2 in plasma was assumed to be 0 5?) The plasma CO_2 content was estimated from whole blood CO_2 content the hemoglobin concentration O_2 saturation and pH of arterial blood using the nomogram of VAN SLYKE and SENDROY (1928) and due correction factors (see PETERS and VAN SLYKE 1931 p 939) P_{aCO} values thus obtained were transformed to body temperature according to BRADLEY STUPFEL and SEVERINGHAUS (1956)

The same procedure was used to estimate *$BHCO_3s$* (the plasma bicarbonate content of blood under standard conditions i e at 37 pH 7 40 and saturated with oxygen)

In this way the $BHCO_3s$ values at rest during CO_2 breathing and during the 1st and 5th–6th min of exercise were obtained The P_{aCO} values during the course of the exercise period were then calculated as described above assuming a linear decrease between the two $BHCO_3s$ values obtained during exercise For the period of recovery the calculation of P_{aCO} was based on the assumption that $BHCO_3s$ again increased inversely with the postulated decay of blood lactic acid i e with a half time of 15 min (cf DILL et al 1935 HENRY and DE MOOR 1950 HUCKABEE 1958)

For the *statistical treatment of data* current conventions have been used for the calculation of arithmetical mean (M) standard deviation (S D) and standard error (S E)

The statistical significance of differences between means was evaluated by applying the t test to the individual differences (cf FISHER 1948) $P < 001$ $P < 01$ $P < 05$ denote highly significant significant and probably significant differences respectively

Subjects and Experimental Procedure

Seven healthy male medical students served as test subjects. The physical characteristics of the subjects are presented in Table I

Each subject participated in three experiments in which the procedure was the same One month s interval was allowed for restoration of hemoglobin lost during the preceding experiment

dead space amounted to 15 ml The *over all external breathing resistance* to a flow of 1 l/sec was 2 6 cm H_2O during inspiration and 0 9 cm H_2O during expiration The CO_2 analyzer was calibrated with five known mixtures of CO_2 in air saturated with water vapor

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VI RESULTS

Introductory Observations

Before describing the time courses obtained for ventilation and for various blood chemical parameters it seems appropriate to communicate other relevant observations which were made during the course of the investigation These concern (1) the validity of end tidal P_{CO} as a measure of the arterial P_{CO} in exercise (2) the effects of artificially induced shifts in the acid base balance on blood $BHCO_{3st}$ and on metabolic rate and heart rate and (3) the ventilatory and blood chemical responses to inhalation of CO_2 which will subsequently be compared with those obtaining in exercise and recovery

End tidal P_{CO} as a Measure of Arterial P_{CO} during Exercise

End tidal gas is generally considered representative of mean alveolar air in the resting supine position During motionless standing end tidal P_{CO} becomes lower than the mean arterial P_{CO_2} (ULMER and REICHEL 1961 BJURSTEDT *et al* 1962) Normal gravity thus produces shifts in the ventilation perfusion ratios within the subdivisions of the lung and an alveolar dead space develops secondary to impaired perfusion of apical regions (*cf* RILEY *et al* 1959)

It seems reasonable to expect that sitting on the bicycle at rest would also produce a measurable alveolar dead space effect That such is the case was borne out by the present experiments in which an average arterial to end tidal CO_2 difference of 2 2 mm Hg was obtained This difference is somewhat lower than that observed by BJURSTEDT *et al* in motionless standing (2 8 mm Hg)

If CO_2 is added to the inspired air the diluting effect of the alveolar dead space ventilation on the expired alveolar air will be diminished In the present investigation this was demonstrated by the fact that the arterial to end tidal P_{CO} difference decreased to practically zero when the subjects sitting quietly on the bicycle inhaled a mixture of 2 8 % CO_2 in air When 6 3 % CO_2 in air was administered the end tidal P_{CO} increased by an average of 15 4 mm Hg whereas the average increase in mean arterial P_{CO} amounted to 12 3 mm Hg As a result of the larger increase in end tidal P_{CO} the arterial to end tidal P_{CO} difference became negative (— 0 9 mm Hg on an average) It is thus evident that the changes in arterial P_{CO} induced by CO_2 breathing would be overestimated if assessed from the changes in end tidal P_{CO} in the sitting or erect position

During exercise the arterial to end tidal P_{CO} difference was also reversed This negative difference was larger than during inhalation of 6 3 % CO_2 averaging — 3 1 mm Hg at the end of exercise The increase in end tidal P_{CO} during the course of exercise averaged 8 4 mm Hg but the concomitant change

In two of these experiments the subject's acid base balance had been altered by ingestion of ammonium chloride and sodium bicarbonate, respectively Either compound was administered orally for three days the daily dosage being 2 5 meq/kg *body weight divided in four doses The last dose was given in the morning* a few hours before the experiment was begun The average daily dose of ammonium chloride was 9 1 g, the average total dose being 27 0 g The average daily dose of sodium bicarbonate was 14 4 g the average total dose being 42 4 g The sequence of experiments was rotated among the subjects

Four to five days before each experiment the subject's health and physical characteristics were assessed The subject was then made thoroughly familiar with the course and various phases of the experiment

All experiments were performed in the morning the subject having previously had a light breakfast The Teflon catheter was introduced percutaneously into the right radial artery under light infiltration anesthesia (BARR 1961) The catheter caused no pain or other inconvenience to the subject who could move his wrist freely with the catheter in position The subject rested in the supine position for half an hour and was given 150 mg Heparin (Vitrum) intravenously to prevent clotting in the pH oximeter sensing unit during the ensuing experiment

The subject then sat up on the bicycle The cardiotachometer electrodes were taped to his chest and the mouth piece was placed in position Finally the radial artery catheter was connected with the pH oximeter recording system The first part of the experiment was devoted to assessing the responses to inhalation of CO_2 while the subject rested on the bicycle For this purpose two different gas mixtures saturated with water vapor were inhaled from plastic Douglas bags, continuously being supplied from large gas cylinders (*cf* Fig 2) The composition of the gas mixtures were the same throughout the investigation and the P_{CO_2} of the inhaled air was checked *at each experiment The subject first inhaled a mixture of 2 8 % CO_2 in air for 10 min* and was then switched over to a mixture of 6 3 % CO_2 in air for another 10 min

The subject then rested for 30 minutes in the supine position breathing air He then was again seated on the bicycle for the second part of the experiment the exercise test He first remained at rest for 10—15 min in the sitting position He was not told when to start working more than a few seconds in advance The subject breathing air (relative humidity 50 %) then pedalled the bicycle for 6 minutes at 50 rpm with a work load of 625 kpm/min and then rested for 6 min while still seated on the bicycle

Sampling of expired gas and of arterial blood for analysis (see Laboratory methods *p 16) was performed during the 9th—10th min* on each gas mixture in the CO_2 test during a 3 min period at rest and during the last 2 min of exercise Blood samples for analysis of CO_2 content were also drawn during the first minute of exercise

On completion of the experiment the subject rested supinely for about *20—30 min* during which period the radial artery catheter was pulled out and the wrist bandaged His physical working capacity at pulse rate 170 (PWC_{170}) was then determined according to SJOSTRAND (1947) and WAHLUND (1948)

Barometric pressure averaged 756 mm Hg (range 748—763 mm Hg) The room temperature of the laboratory was kept within 1 C and averaged 24 4 C the relative humidity being about 50 %

VI RESULTS

Introductory Observations

Before describing the time courses obtained for ventilation and for various blood chemical parameters it seems appropriate to communicate other relevant observations which were made during the course of the investigation These concern (1) the validity of end tidal P_{CO} as a measure of the arterial P_{CO} in exercise (2) the effects of artificially induced shifts in the acid base balance on blood $BHCO_{3st}$ and on metabolic rate and heart rate and (3) the ventilatory and blood chemical responses to inhalation of CO_2 which will subsequently be compared with those obtaining in exercise and recovery

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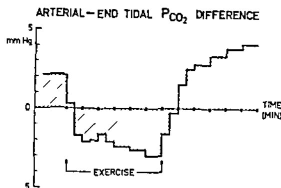


Fig 3 The arterial to end tidal P_{CO_2} difference during rest exercise (625 kpm/min for 6 min) and recovery Data obtained from continuous recordings (from 16 experiments in 7 subjects) have been reduced into group means of individual time averages determined over the first six 30-sec periods in exercise and over the first four 30-sec periods in recovery Other time averages refer to 60-sec periods.

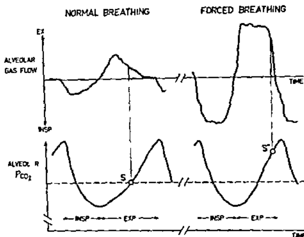
in arterial blood P_{CO} *was only 3 1 mm Hg* The time course of the observed mean changes in arterial to end tidal P_{CO} difference during exercise and recovery is depicted graphically in Fig 3 *The reversal of the gradient occurred* at the beginning of exercise, during recovery the gradient returned to the pre exercise resting value within 2 min

It is evident then that even breath by breath recordings of end tidal P_{CO} do not give a true picture of the dynamic changes in P_{aCO} resulting from exercise This observation is in agreement with the statement of KROGH and LINDHARD (1913a) that the direct method of determining the composition of the alveolar air from samples taken at the end of inspiration and expiration becomes untrustworthy during muscular work

Direct determinations during exercise of both end tidal (end expiratory) and arterial P_{CO_2} in the same individuals have been made by only a few investigators SUSKIND *et al* (1950) concluded that, on the whole, end tidal and arterial P_{CO_2} changed in a more or less parallel fashion during exercise In their Table 2, however, it can be seen that the arterial to end tidal P_{CO} difference was $+2\,1$ mm Hg at rest, and $-1\,8$ mm Hg during exercise ($V_O = 0\,596$ l/m²) In a study on the respiratory dead space during exercise ASMUSSEN and NIELSEN (1956) found that during exercise with large tidal volumes (*work load 360—1,080 kpm/min) the* P_{CO} *of end expiratory* samples was higher than that of simultaneously drawn arterial blood samples

The observation of a reversed P_{CO} difference during forced breathing as during CO_2 inhalation and exercise may be explained as follows The end tidal sampler used in this investigation (BRISMAR HESSER and MATELL 1962) as well as that described by RAHN *et al* (1946) automatically delivers a small sample from a portion of expired air just *beyond the expiratory valve* The small amount of gas sampled leaves the alveolar compartment within a very short period of time during expiration and its composition relative to the mean alveolar air will thus be dependent both on the moment of sampling and on the concomitant slope of the alveolar P_{CO} curve (*cf* Fig 4) (*cf* RAHN 1955)

Fig 4 Schematical representation of time-courses of alveolar gas flow and Pco during a respiratory cycle for normal and forced breathing Hatched areas represent the portion of alveolar gas that fills the dead space at the end of an expiration (including dead space of valve) In normal breathing the Pco of the end tidal sample (S') approximates that of mean alveolar air In forced breathing the end tidal sample (S) is drawn close to the end of expiration and will thus yield an end tidal Pco that is higher than the mean alveolar Pco



The moment of sampling will in turn be dependent on the dead space/tidal volume ratio as well as on the flow pattern during expiration This is schematically illustrated in Fig 4 The upper part of the figure shows the rate of flow of the alveolar gas as it leaves the alveolar compartment The lower part of the figure shows the time course of $P_{A_{CO}}$ during a respiratory cycle and its mean value

During *quiet breathing* the midpoint of the period of sampling (S') falls near the middle of the time interval between minimal and maximal values of $P_{A_{CO}}$ During *forced breathing* induced *e g* by CO_2 breathing or exercise the pattern of gas flow from the alveolar compartment *versus* time will be changed (TIITSO 1935 CAIN and OTIS 1949) so that the time required to fill the dead space with alveolar gas at the end of expiration will be much shorter than during quiet breathing This is the same as saying that the CO_2 molecules arriving beyond the expiratory valve at the end of the expiration have travelled more rapidly through the dead space Hence, the interval between the time during which these molecules left the alveolar compartment (S) and the time for maximal $P_{A_{CO}}$ must be shortened The final result will then be that the end tidal P_{CO_2} becomes higher than the arterial or mean alveolar P_{CO} approximating the maximal alveolar P_{CO} occurring during the respiratory cycle

The slope of the alveolar P_{CO} curve during expiration is another factor that determines the magnitude of the difference between end tidal P_{CO} and mean $P_{A_{CO}}$ This difference will be intimately related to the magnitude of the cyclic variations of alveolar P_{CO} within the respiratory cycle DUBOIS BRITT and FENN (1952) have described the theoretically expected variations at rest on which the left part of the diagram in Fig 4 is based During exercise much larger variations do occur as has been theoretically predicted by YAMA

Table II Oxygen uptake, heart rate, standard bicarbonate and respiratory quotient at rest and

Conditions *NB* = normal *HB* = high $BHCO_{3\,St}$ *LB* = low $BHCO_{3\,St}$

	Oxygen uptake (l STPD/min)			Heart rate (beats/min)		
	Rest[1]	Exercise[2]	Difference Exercise—Rest	Rest[1]	Exercise[2]	Difference Exercise—Rest
NB						
M ± S E	311 ± 011	1 587 ± 044	+ 1 275 ± 037	78 ± 4	131 ± 5	+53 ± 4
S D	030	115	099	11	13	12
P			< 001			< 001
HB – NB						
M ± S E	− 014 ± 017	+ 015 ± 084	+ 030 ± 074	−8 ± 5	−4 ± 6	+5 ± 6
P	> 05	> 05	> 05	> 05	> 05	> 05
LB – NB						
M ± S E	+ 009 ± 016	+ 032 ± 034	+ 023 ± 028	+7 ± 3	+5 ± 5	−2 ± 3
P	> 05	> 05	> 05	> 05	> 05	> 05

[1] Mean value over 3 min

[2] Mean of 5th and 6th min after onset of exercise

MOTO (1960) BUHLMANN (1961) *has measured the increasing variations in the* expired P_{CO} during exhausting exercise, and points out the difficulties in assessing the composition of the mean alveolar air during heavy exercise In *the present investigation the arterial to end tidal* P_{CO} *difference amounted* to — 3 1 mm Hg in the steady state of ventilation during exercise The tidal and respiratory minute volumes were about the same as in experiments with inhalation of 6 3 % CO_2 (*cf* above) *Hence, the displacement in time of the* alveolar gas sampling (S′) would be approximately the same in the exercise and CO_2 experiments It may be inferred then that the larger part of the negative arterial to end tidal P_{CO} difference during exercise would be due to the increasing variations in P_{ACO} within the respiratory cycle

Theoretical calculations, similar to those of YAMAMOTO (*cf* above) have been performed in this investigation using the measured values for ventilation and gas exchange, and assuming the measured end tidal P_{CO} to be an approximation of the maximal P_{CO} during the respiratory cycle in forced breathing The calculated difference between mean alveolar P_{CO} and the end tidal

during exercise (n = 7 except when otherwise indicated)

Standard bicarbonate (mM/l)			Respiratory quotient		
Rest	Exercise[1]	Difference Exercise—Rest	Rest[1]	Exercise	Difference Exercise—Rest
46 04	24 17	− 1 87	777	.892	+ 114
± 65	± .87	± .33	± 006	± 073	± 071
1 71	2.29	88	015	060	055
		< 01			< 01
+ 3 36	+ 2 65	− 71	+ 003	+ 009	+ 005
± .36	± 40	± .28	± 022	± 017	± 019
< 001	< 001	< 05	> 05	> 05	> 05
− 9.51	− 9 52	− 02	− 001	− 019	− 018
± 1 13	± 1.33	± 40	± 015	± 017	± 028
< .001	< 001	> 05	> 05	> 05	> 05

* Plasma bicarbonate content of blood under standard condition i e at 37 C, pH 7 40 and saturated with oxygen.
n = 6

P_C was found to be in good agreement with the directly measured arterial to end tidal P_{CO} difference during inhalation of 6 3 % CO_2 the measured difference was — 0 9 mm Hg and the calculated difference — 1 0 mm Hg During exercise the measured difference was — 3 1 mm Hg and the calculated difference — 4 6 mm Hg

It may be said in conclusion that the present observation of a negative arterial to end tidal CO_2 difference during exercise and during inhalation of CO_2 necessitates caution when attempting to estimate the level of the arterial P_{CO} (or mean alveolar P_{CO}) from measurements of the P_{CO} of end tidal samples This is even more the case in the transition from normal quiet breathing when a positive arterial to end tidal CO_2 difference exists because of an appreciable alveolar dead space This is one reason why in the present studies of time related changes in arterial P_{CO} during exercise and recovery reliance was placed on P_{aCO} values calculated from the arterial pH recordings rather than on end tidal P_{CO} recordings

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P	> 05	> 05	> 05	> 05	> 05	> 05
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M ± S E	+ 009 ± 016	+ 032 ± 034	+ 023 ± 028	+7 ± 3	+5 ± 5	–2 ± 3
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		< 01			< 01
+ 3 36	+ 2 65	− 71	+ 003	+ 009	+ 005
± 36	± 40	± 28	± 072	± 017	± 019
< 001	< 001	< 05	> 05	> 05	> 05
− 9.51	− 9 52	− 02	− 001	− 019	− .018
± 1 13	± 1.33	± 40	± 015	± 017	± 078
< 001	< 001	> 05	> .05	> 05	> 05

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Effects of Artificially Induced Shifts in the Acid Base Balance on Oxygen Uptake, Heart Rate and Blood Bicarbonate

The work load was kept the same throughout the investigation in order to make possible a comparison between the ventilatory and blood chemical responses to exercise in conditions where shifts in the acid base balance had been artificially induced An account will be given below of the effects on the oxygen uptake, heart rate, standard bicarbonate and respiratory quotient observed in conditions of normal, high and low $BHCO_{3St}$, here termed "NB", "HB" and "LB", respectively The changes observed are summarized in Table II

Oxygen uptake The deviations observed in O_2 uptake at rest in LB and HB were small and statistically insignificant During the 5th—6th min of exercise the O_2 uptake had risen to an average of about 1 6 l/min No significant differences were found between the three conditions This was the case also for the increases in O_2 uptake as expressed per m^2 body surface

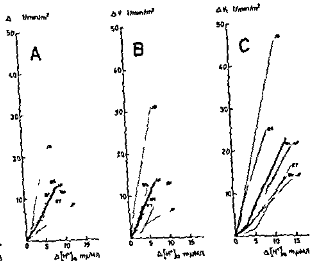
Heart rate At rest, the differences between NB and HB and between NB and LB were not statistically significant The difference between HB and LB, however, was significant (not shown in Table II) By contrast, the increases in heart rate during exercise above resting values were similar in the three conditions

Blood Bicarbonate The shift in $BHCO_{3St}$ (expressed in mM/l) observed at rest after ingestion of ammonium chloride was more than twice that obtained after ingestion of equivalent amounts of sodium bicarbonate During exercise a certain amount of lactic acid was presumably produced (*cf* HUCKABEE 1958, HOLMGREN and STROM 1959), which caused a reduction of the $BHCO_{3St}$ in all three conditions These reductions were statistically significant The decrease was slightly larger in HB than in LB and NB The $BHCO_{3St}$ was also determined during the first minute of exercise The average decrease during the first minute in all three conditions was 0 34 mM/l ($P < 0\ 001$)

From the data presented above it can be concluded that the period of exercise (625 kpm/min for 6 min) involved about the same load in conditions NB HB and LB It is probable, however, *that if the $BHCO_{3St}$ shifts had been* more pronounced, an impairment in working performance would have resulted (*cf* the experiments of DENNIG *et al* (1930) and RERSUM (1961))

Effects of Acid Base Displacements on the $\Delta V_I/\Delta[H^+]_a$ and $\Delta V_I/\Delta P_{aCO}$ Slopes Resulting from CO_2 Inhalation

The results of inhalation of CO_2 air mixtures at rest in conditions NB HB and LB are summarized in Fig 5 The oxygen fractions in the inspired air were chosen so as to minimize recorded changes in O_2 saturation of the arterial blood during the hyperpnea that resulted from inhalation of CO_2 enriched air The O_2 fraction was 0 18 in the gas with the low CO_2 fraction (0 028) and 0 16 in the gas with the higher CO_2 fraction (0 063) As can be seen in Tables III



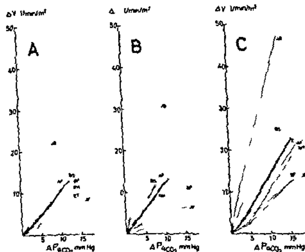


Fig 5 Individual increments (from resting levels) in ventilation plotted against increments in arterial hydrogen ion concentration (upper 3 graphs) and in arterial carbon dioxide tension (lower 3 graphs)

Values were obtained during 9th and 10th min under inhalation of gas mixtures containing 2 8 and 6 3 per cent CO_2 in air

Heavy lines indicate means of individual $\Delta V/\Delta[H^+]$ and $\Delta V/\Delta P_{aCO}$ slopes (*thin lines*)

A B and C denote normal, high and low $BHCO_{3St}$ respectively

and IV the O_2 saturation remained almost constant during CO_2 inhalation, whether studied in the NB HB or LB conditions The O_2 tension however showed greater changes and an increased O_2 tension was evident during inhalation of 6 3 % CO_2

$\Delta V_E/\Delta[H^+]$ *Slopes* (upper graphs in Fig 5) The mean slopes were similar in the three conditions A closer look at the upper three graphs in Fig 5 reveals that the individual slope was nearly the same in all three conditions although the absolute increments in ventilation and $[H^+]$ were greater in HB and more so in LB than in NB On the average an increase of $[H^+]$

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and 6 3 CO_2 in air) n = 7

$[H^+]$ [1.5] (mμM/l)		P_{O_2} [1.5] (mm Hg)		P_{CO_2} [1.4] (mm Hg)	
2.8 CO_2	6.3 CO_2	2.8% CO_2	6.3 CO_2	2.8 CO_2	6.3 CO_2
38.8	45.7	88.8	101.7	40.4	50.2
± .6	± .7	± 1.5	± 2.1	± 1.0	± .8
1.7	1.9	4.0	5.4	2.7	2.2
36.0	41.5	83.0	96.5	40.9	49.7
± .6	± .9	± 1.3	± 2.3	± 1.1	± 1.3
1.7	2.3	3.3	5.9	3.0	3.3
47.2	57.6	97.0	104.3	34.8	47.3
± 1.6	± 1.7	± 2.7	± 3.5	± .6	± .7
4.2	4.5	7.1	9.2	1.5	1.7
− 2.8	− 4.1	− 5.8	− 5.2	+ .5	− .5
± .5	± .5	± 1.6	± 2.2	± 1.0	± 1.0
< .01	< .001	< .01	> .05	> .05	> .05
+ 8.4	+ 11.8	+ 8.2	+ 2.6	− 5.6	− 2.9
± 1.3	± 1.9	± 4.1	± 5.3	± .8	± 1.0
< .01	< .001	> .05	> .05	< .001	< .05

Calculated from O_2-saturation and pH by use of the line charts of SEVERINGHAUS (1958)
Computed from the HENDERSON HASSELBALCH equation

Absolute mean values of the parameters studied in the 9th and 10th min of the CO_2 inhalation experiments are given in Table III

In summary the experiments with CO_2 breathing at two different levels of inhaled P_{CO_2} (2 8 and 6 3 % CO_2 in air) showed that the resulting ventilatory increment as studied in normal high and low $BHCO_{3s}$ was better correlated with the concomitant increment in $[H^+]$ than with that of P_{CO_2} for the individual as well as for the group

Table III Data obtained in 9th and 10th min of experiments on CO_2 breathing at rest (2 8 %
Conditions *NB* = normal *HB* = high $BHCO_3$ *LB* = low $BHCO_{3St}$

	V_I (1 BTPS/min/m²)		V_T (1 BTPS/m²)		S_{aO} (%)	
	2 8 % CO_2	6 3 % CO_2	2 8 % CO_2	6 3 % CO_2	2 8 % CO_2	6 3 % CO_2
NB						
M ± S E.	6 34	17 37	445	950	96 1 ± 1	96 6 ± 1
	± 21	± 1 71	± 022	± 074		
S D	54	4 53	059	196	3	4
HB						
M ± S E	6 15	17 08	471	1 021	95 9 ± 2	96 6 ± 2
	± 36	± 3 19	± 014	± 091		
S D	95	8 43	036	242	5	4
LB						
M ± S E.	7 95	27 84	576	1 357	96 2 ± 2	96 1 ± 2
	± 43	± 4 16	± 043	± 081		
S D	1 15	11 0	114	214	5	6
HB − NB						
M ± S E	− 18	− 27	+ 026	+ 071	− 2 ± 2	0 ± 2
	± 39	± 1 50	± 016	± 023		
P	> 05	> 05	> 05	< 05	> 05	> 05
LB − NB						
M ± S E	+ 1 61	+ 10 49	+ 131	+ 408	+ 1 ± 2	− 5 ± 3
	± 41	± 2 62	± 033	± 038		
P	< 01	< 01	< 01	< 001	> 05	> 05

[1] At body temperature
[2] $[H^+]_a$ = hydrogen ion concentration of arterial blood in mμM/l
35 mμM/l = pH 7 456 40 mμM/l = pH 7 398 45 mμM/l = pH 7 347 50 mμM/l = pH 7 301

by one mμM/l was associated with a ventilatory increase of 2 0 l/m² BSA in the three conditions

$\Delta V_I/\Delta P_{aCO_2}$ *Slopes* (lower graphs in Fig 5) The mean slope was found to be steeper in LB than in the other two conditions Otherwise it can be seen from the lower graphs in Fig 5, that the individual slopes show approximately the same sequence in the families of curves for the three conditions The average ventilatory increase per mm Hg increase in P_{CO_2} was 1 3 l/m² in NB 1 4 l/m² in HB and 1 7 l/m² in LB

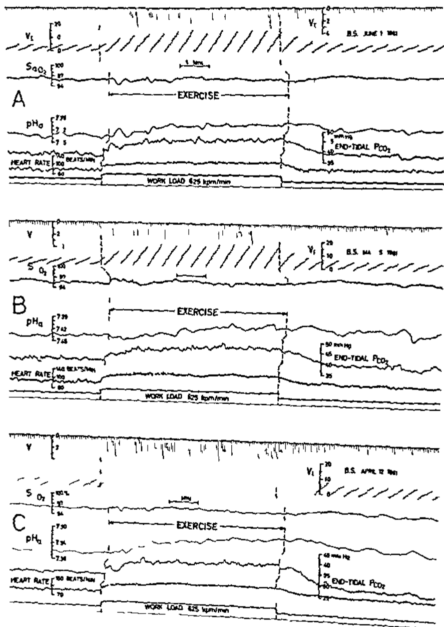


Fig 6 Set of photokymographic recordings of responses in subject *BS* to constant load exercise (625 kpm/min for 6 minutes) under conditions of normal $BHCO_{3St}$ (A) high $BHCO_{3St}$ (B) and low $BHCO_{3St}$ (C) (length of records = *1/10 of* original)

Dashed lines (isotime lines) indicate the delays in the S_{aO_2} pH and end tidal P_{CO_2} tracings.

Time-Courses of Ventilatory and Blood Chemical Responses to Constant-Load Exercise

In Fig 7 the time courses of different parameters during constant load, dynamic exercise (625 kpm/min) and recovery are depicted graphically as obtained in the conditions of normal, high and low $BHCO_{3s}$. The graphs were based on data gathered from records as exemplified in Fig 6 Absolute values (means) at rest and during exercise are given in Table IV

Inspired Minute Volume At the onset of exercise ventilation increased almost immediately The initial rise was more rapid in HB and LB than in NB

The initial rise was followed by a slower secondary increase, which continued until a plateau was attained within 3—4 min The mean values in the 5th and 6th min (*cf* Table IV) indicate that the ventilation became significantly greater in LB than in HB or NB, and that the difference between NB and HB was not significant

At the cessation of exercise there was a sudden fall of ventilation followed by a smooth decay, the shape of which was similar in the three conditions In the sixth minute of recovery the ventilation was increas[illegible] less than one liter/m² BSA above its pre-exercise level.

$[H^+]_a$ Fig 7 shows the time-courses of individual and m[illegible] values, which were obtained from the arterial pH recordin[illegible] Du[illegible] minute of exercise $[H^+]_a$ remained essentially con[illegible] in HB and LB Subsequently, the general trend was[illegible] in all three conditions The absolute, mean valu[illegible] during the 5th and 6th min of exercise in NB, I[illegible] Table IV

At the cessation of exercise the mean $[H^+]$ remai[illegible] or increased somewhat during the first half minute i[illegible] and then decreased at a diminishing rate until the[illegible] period of recovery when $[H^+]_a$ still remained well abov[illegible]

S_{a_O} and P_{a_O} In the original recordings the arterial O[illegible] remarkably constant throughout the exercise and recove[illegible] Small but consistent changes could, however, be detec[illegible] ginning of exercise, the S_{a_O} first increased somewhat a[illegible] resting level to a minimum, which was attained af[illegible] then increased again, so that within the 5th and 6th mi[illegible] was reached (*cf* Table IV) At the cessation of exercise[illegible] for about 30 sec. During recovery S_{a_O} rose to a max[illegible] after exercise and then declined again. This increase[illegible] amounted to an average of abo[illegible]

The time-course of the [illegible] owed [illegible] as described above for S_{a_O} [illegible] Fig 7 A[illegible] the onset of ex[illegible]

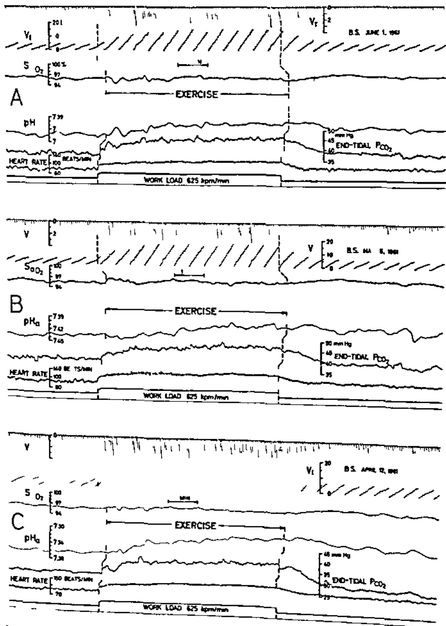


Fig 6 Set of photokymographic recordings of responses in subject *BS* to constant load exercise (625 kpm/min for 6 minutes) under conditions of normal $BHCO_{3st}$ (A) high $BHCO_{3st}$ (B) and low $BHCO_{3st}$ (C) (length of records = 1/10 of original)
Dashed lines (isotime lines) indicate the delays in the S_{O_2} pH and end tidal P_{CO_2} tracings

Time-Courses of Ventilatory and Blood Chemical Responses to Constant-Load Exercise

In Fig 7 the time courses of different parameters during constant load, dynamic exercise (625 kpm/min) and recovery are depicted graphically as obtained in the conditions of normal, high and low $BHCO_{3st}$ The graphs were based on data gathered from records as exemplified in Fig 6 Absolute values (means) at rest and during exercise are given in Table IV

Inspired Minute Volume At the onset of exercise ventilation increased almost immediately The initial rise was more rapid in HB and LB than in NB

The initial rise was followed by a slower secondary increase, which continued until a plateau was attained within 3—4 min The mean values in the 5th and 6th min (*cf* Table IV) indicate that the ventilation became significantly greater in LB than in HB or NB and that the difference between NB and HB was not significant

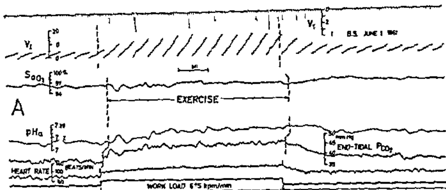
At the cessation of exercise there was a sudden fall of ventilation followed by a smooth decay, the shape of which was similar in the three conditions In the sixth minute of recovery the ventilation was increased by less than one liter/m² BSA above its pre exercise level

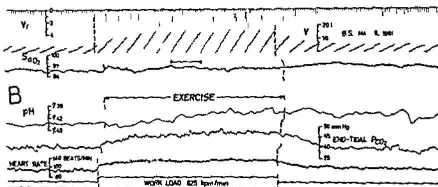
$[H^+]_a$ Fig 7 shows the time courses of individual and mean $[H^+]_a$ values, which were obtained from the arterial pH recordings During the first half minute of exercise $[H^+]_a$ remained essentially constant in NB as well as in HB and LB Subsequently, the general trend was towards an acidotic shift in all three conditions The absolute, mean values for $[H^+]_a$ at rest and during the 5th and 6th min of exercise in NB, HB and LB are given in Table IV

At the cessation of exercise the mean $[H^+]_a$ remained essentially constant or increased somewhat during the first half minute in all three conditions, and then decreased at a diminishing rate until the end of the recorded period of recovery when $[H^+]_a$ still remained well above the pre exercise level

S_{aO_2} *and* P_{aO_2} In the original recordings, the arterial O_2 saturation remained remarkably constant throughout the exercise and recovery periods (*cf* Fig 6) Small but consistent changes could, however be detected Thus, at the beginning of exercise, the S_{aO_2} first increased somewhat and then fell below the resting level to a minimum, which was attained after some 60—90 sec S_{aO_2} then increased again, so that within the 5th and 6th min the pre exercise level was reached (*cf* Table IV) At the cessation of exercise S_{aO_2} remained constant for about 30 sec During recovery S_{aO_2} rose to a maximum about 2 minutes after exercise and then declined again This increase above the resting level amounted to an average of about 1 %

The time course of the oxygen tension showed the same general pattern as described above for S_{aO_2} but the changes were considerably more marked (Fig 7) At the onset of exercise P_{aO_2} remained unchanged in the first half





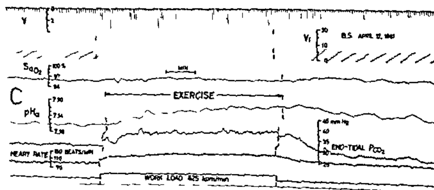


Fig 6 Set of photokymographic recordings of responses in subject *BS* to constant load exercise (6 5 kpm/min for 6 minutes) under conditions of normal $BHCO_{3st}$ (A) high $BHCO_{3st}$ (B) and low $BHCO_{3st}$ (C) (length of records = 1/10 of original)

Dashed lines (isotime lines) indicate the delays in the S_{aO_2}, pH_a and end tidal P_{CO_2} tracings

Time-Courses of Ventilatory and Blood Chemical Responses to Constant-Load Exercise

In Fig 7 the time courses of different parameters during constant load, dynamic exercise (625 kpm/min) and recovery are depicted graphically as obtained in the conditions of normal, high and low $BHCO_{3st}$ The graphs were based on data gathered from records as exemplified in Fig 6 Absolute values (means) at rest and during exercise are given in Table IV

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At the cessation of exercise there was a sudden fall of ventilation followed by a smooth decay, the shape of which was similar in the three conditions *In the sixth minute of recovery the ventilation* was increased by *less than* one liter/m² BSA above its pre exercise level

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At the cessation of exercise the mean $[H^+]_a$ remained essentially constant or increased somewhat during the first half minute in all three conditions, and then decreased at a diminishing rate until the end of the recorded period of recovery when $[H^+]_a$ still remained well above the pre exercise level

S_{aO_2} and P_{aO_2} In the original recordings, the arterial O_2 saturation remained remarkably constant throughout the exercise and recovery periods (*cf* Fig 6) Small but consistent changes could, however, be detected Thus at the beginning of exercise, the S_{aO_2} first increased somewhat and then fell below the resting level to a minimum, which was attained after some 60—90 sec S_{aO_2} then increased again, so that within the 5th and 6th min the pre exercise level was reached (*cf* Table IV) At the cessation of exercise S_{aO_2} remained constant for about 30 sec During recovery S_{aO_2} rose to a maximum about 2 minutes after exercise and then declined again This increase above the resting level amounted to an average of about 1 %

The time course of the oxygen tension showed the same general pattern *as described above for* S_{aO_2}, but the changes were considerably more marked (Fig 7) At the onset of exercise P_{aO_2} remained unchanged in the first half

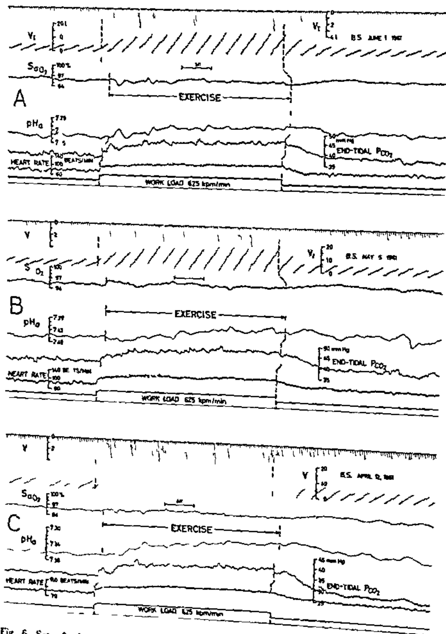


Fig 6 Set of photokymographic recordings of responses in subject *BS* to constant load exercise (625 kpm/min for 6 minutes) under conditions of normal $BHCO_{3se}$ (A) high $BHCO_{3se}$ (B) and low $BHCO_{3se}$ (C) (length of records = 1/10 of original)
Dashed lines (isotime lines) indicate the delays in the S_{aO_2}, pH and end tidal P_{CO_2} tracings.

Time-Courses of Ventilatory and Blood Chemical Responses to Constant-Load Exercise

In Fig 7 the time courses of different parameters during constant load, dynamic exercise (625 kpm/min) and recovery are depicted graphically as obtained in the conditions of normal, high and low $BHCO_{3st}$. The graphs were based on data gathered from records as exemplified in Fig 6 Absolute values (means) at rest and during exercise are given in Table IV

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The initial rise was followed by a slower secondary increase, which continued until a plateau was attained within 3—4 min The mean values in the 5th and 6th min (*cf* Table IV) indicate that the ventilation became significantly greater in LB than in HB or NB, and that the difference between NB and HB was not significant

At the cessation of exercise there was a sudden fall of ventilation followed by a smooth decay, the shape of which was similar in the three conditions In the sixth minute of recovery the ventilation was increased by less than one liter/m² BSA above its pre exercise level

$[H^+]_a$ Fig 7 shows the time courses of individual and mean $[H^+]_a$ values, which were obtained from the arterial pH recordings During the first half minute of exercise $[H^+]$ remained essentially constant in NB as well as in HB and LB Subsequently, the general trend was towards an acidotic shift in all three conditions The absolute, mean values for $[H^+]_a$ at rest and during the 5th and 6th min of exercise in NB, HB and LB are given in Table IV

At the cessation of exercise the mean $[H^+]_a$ remained essentially constant or increased somewhat during the first half minute in all three conditions and then decreased at a diminishing rate until the end of the recorded period of recovery when $[H^+]_a$ still remained well above the pre exercise level

$S_{a_{O}}$ and $P_{a_{O}}$ In the original recordings, the arterial O_2 saturation remained remarkably constant throughout the exercise and recovery periods (*cf* Fig 6) Small but consistent changes could, however, be detected Thus, at the beginning of exercise, the S_{O} first increased somewhat and then fell below the resting level to a minimum, which was attained after some 60—90 sec S_{a_O} then increased again, so that within the 5th and 6th min the pre exercise level was reached (*cf* Table IV) At the cessation of exercise S_{O} remained constant for about 30 sec During recovery S_{a_O} rose to a maximum about 2 minutes after exercise and then declined again This increase above the resting level amounted to an average of about 1 %

The time course of the oxygen tension showed the same general pattern as described above for S_{a_O}, but the changes were considerably more marked (Fig 7) At the onset of exercise $P_{a_{O_2}}$ remained unchanged in the first half

B

$\Delta \dot{V}_I$ l/min/m²

$\Delta[H^+]_a$ mμM/l

ΔP_{aCO_2} mm Hg

ΔP_{aO_2} mm Hg

EXERCISE RECOVERY

0 2 4 6 8 10 12 MIN

C

$\Delta \dot{V}_I$ l/min/m²

$\Delta[H^+]_a$ mμM/l

ΔP_{aCO_2} mm Hg

ΔP_{aO_2} mm Hg

EXERCISE RECOVERY

0 2 4 6 8 10 12 MIN

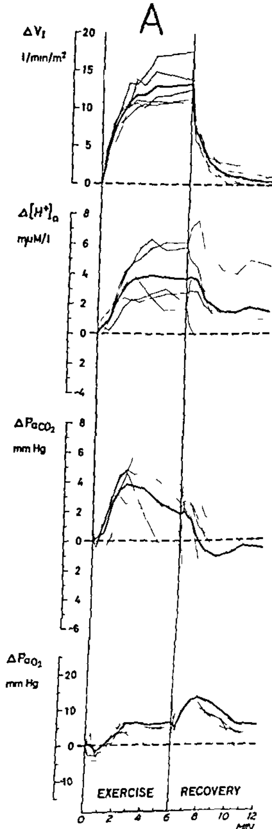


Fig 7 Time courses of deviations from resting values in (from above) V_I $[H^+]$ P_{aCO_2} and P_{aO_2} during exercise (625 kpm/min for 6 minutes) and recovery under normal $BHCO_{3St}$ (A, n = 5) high $BHCO_{3St}$ (B n = 5) and low $BHCO_{3St}$ (C n = 6)

Heavy lines show means of individual time averages (*thin lines*) determined over successive 30 sec periods (first 3 min of exercise and first 2 min of recovery) and 60 sec periods (remaining phases of exercise and recovery)

In plotting the time courses for blood chemical changes due allowance has been made for the delays *in the recordings* (*cf* Fig 6)

$[H^+]$ [3 4] (mμM/l)			P_{O_2} [3 4] (mm Hg)			P_{CO_2} [3 4] (mm Hg)		
Rest	Exercise	Difference Exercise—Rest	Rest[1]	Exercise	Difference Exercise—Rest	Rest	Exercise[4]	Difference Exercise—Rest
37 3	41.3	+ 4 0	87 0	9? 1	+ 5 1	37 9	41 0	+ 3 1
± 7	± 1 0	± 7	± 1 8	± 2 4	± 1.9	± 1 1	± 9	± .8
1 9	2 7	1 8	4 7	6 4	5 3	2 9	2 4	2 1
		< 01			< 05			< 01
34 7	38 5	+ 3.8	82 6	87 7	+ 5 1	38 7	41 0	+ 2 3
± 4	± 7	± 5	± 1 9	± 2 3	± 1 9	± .9	± 1 2	± 7
1 2	1 7	1 3	5 0	6 0	5 0	2.5	3 0	1 8
		< 001			< 05			< 05
44.2	49 8	+ 5 6	95 5	99 1	+ 3 6	32 0	35 3	+ 3.3
± 1 4	± 1 0	± 1 0	± 1.5	± 2 5	± 1 5	± 9	± 9	± 1.2
3 8	2 5	2 6	3 9	6 7	3 9	2 4	2 5	3 2
		< 01			< 05			< 05
− 2 6	− 2 7	− 1	− 4 4	− 4 4	− 1	+ 8	+ 1	− 8
± 7	± 7	± .2	± 1 9	± 2.5	± 1 7	± 9	± 1 0	± 4
< 01	< 01	> 05	> 05	> 05	> 05	> 05	> 05	> 05
+ 6 9	+ 8 6	+ 1 7	+ 8.5	+ 7 0	− 1 5	− 5 9	− 5 7	+ .2
± 1 6	± 1 4	± .8	± 2 9	± 3 7	± 2 3	± 6	± 1 0	± 1 1
< 01	< 001	> 05	< 05	> 05	> 05	< 001	< 01	> 05

$[H^+]$ = hydrogen ion concentration of arterial blood in mμM/l
35 mμM/l = pH 7 456   40 mμM/l = pH 7 398   45 mμM/l = pH 7 347   50 mμM/l = pH 7.301
Calculated from O_2 saturation and pH by use of the line charts of SEVERINGHAUS (1958)
Computed from the HENDERSON HASSELBALCH equation.

Table II Data obtained at rest and during exercise (n = 7)

Conditions NB = normal HB = high LB = low $BHCO_{3St}$

	V_I (l BTPS/min/m²)			V_T (l BTPS/m²)			S_{aO_2} (%)		
	Rest[1]	Exercise[2]	Difference Exercise—Rest	Rest[1]	Exercise[2]	Difference Exercise—Rest	Rest[1]	Exercise[2]	Difference Exercise—Rest
NB									
M ± S E	4 33	17 19	+ 12 86	318	943	625	96 1	96 1	+ 1
	± 15	± 84	± 89	± 020	± 051	± 042	± 1	± .2	± .2
S D	39	2.23	2 35	053	135	111	.2	6	5
P			< 001			< 001			> 05
HB									
M ± S E	3 90	17 12	+ 13 22	315	900	.585	95 9	96 0	+ 1
	± 12	± 1 03	± 97	± 012	± 052	± 053	± .2	± 2	± 2
S D	32	2 72	2.56	032	138	141	5	6	6
P			< 001			< 001			> 05
LB									
M ± S E	5 12	20 13	+ 15 00	349	1 033	685	96 3	96 1	− .2
	± 36	± 87	± 1 07	± 022	± 062	± 050	± 1	± 2	± 2
S D	95	2 30	2 84	058	164	132	3	6	6
P			< 001			< 001			> 05
HB — NB									
M ± S E.	− 43	− 07	+ 36	− 003	− 043	− 040	− 1	− 1	0
	± 15	± 68	± 65	± 012	± 065	± 059	± .2	± 2	± 2
P	< 05	> 05	> 05	> 05	> 05	> 05	> 05	> 05	> 05
LB — NB									
M ± S E	+ 80	+ 2 94	+ 2 14	+ 030	+ 090	+ 060	+ 2	0	− 3
	± 31	± 62	± 90	± 030	± 068	± [illegible]	2	± 3	± 3
P	< 05	< 01	> 05	> 05	> 05	> [illegible]	[illegible]	[illegible]	> 05

[1] Mean value over 3 min

[2] Mean of 5th—6th min of exercise.

[3] At body temperature.

$[H^+]$[3,4] ($m\mu M/l$)			P_{O_2}[3,5] (mm Hg)			P_{CO_2}[3,6] (mm Hg)		
Rest	Exercise	Difference Exercise—Rest	Rest[1]	Exercise	Difference Exercise—Rest	Rest[1]	Exercise[2]	Difference Exercise—Rest
37 3 ± 7 1 9	41.3 ± 1 0 2 7	+ 4 0 ± 7 1 8 < 01	87 0 ± 1.8 4 7	92 1 ± 2 4 6 4	+ 5 1 ± 1 9 5.3 < 05	37 9 ± 1 1 2.9	41 0 ± 9 2 4	+ 3 1 ± .8 2 1 < 01
34 7 ± 4 1.2	38 5 ± 7 1 7	+ 3.8 ± .5 1 3 < 001	82 6 ± 1 9 5 0	87 7 ± 2 3 6 0	+ 5 1 ± 1.9 5 0 < 05	38 7 ± .9 2.5	41 0 ± 1.2 3 0	+ 2.3 ± 7 1.8 < 05
44 2 ± 1 4 3 8	49 8 ± 1 0 2 5	+ 5 6 ± 1 0 2 6 < 01	95 5 ± 1.5 3 9	99 1 ± 2.5 6 7	+ 3 6 ± 1.5 3.9 < 05	32 0 ± 9 2 4	35 3 ± .9 2 5	+ 3.3 ± 1.2 3.2 < 05
− 2 6 ± 7 < 01	− 2 7 ± 7 < 01	− 1 ± .2 > 05	− 4 4 ± 1 9 > 05	− 4 4 ± 2 5 > 05	− 1 ± 1 7 > 05	+ 8 ± .9 > 05	+ 1 ± 1 0 > 05	− .8 ± 4 > 05
+ 6 9 ± 1 6 < 01	+ 8 6 ± 1 4 < 001	+ 1 7 ± .8 > 05	+ 8 5 ± 2 9 < 05	+ 7 0 ± 3 7 > 05	− 1.5 ± 2.3 > 05	− 5 9 ± 6 < 001	− 5 7 ± 1 0 < 01	+ .2 ± 1 1 > 05

[4] $[H^+]$ = hydrogen ion concentration of arterial blood in $m\mu M/l$
35 $m\mu M/l$ = pH 7 456 40 $m\mu M/l$ = pH 7 398 45 $m\mu M/l$ = pH 7 347 50 $m\mu M/l$ = pH 7 301

[5] Calculated from O_2 saturation and pH by use of the line charts of SEVERINGHAUS (1958)

[6] Computed from the HENDERSON HASSELBALCH equation.

minute, showing a decrease in the second half minute $P_{a_{O_2}}$ then increased above its pre exercise level and remained 4—5 mm Hg higher than in the pre exercise period During the first half minute of recovery $P_{a_{O_2}}$ remained unchanged, and then rose sharply The rise during recovery was more marked in HB than in NB or LB After attaining its maximum in the second minute of recovery, $P_{a_{O_2}}$ again rapidly declined

$P_{a_{CO_2}}$ *The mean time course of the calculated* $P_{a_{CO_2}}$ approximated that of the arterial $[H^+]$ at the very beginning of exercise In their further courses the two variables diverged, however, due to a decrease of the $BHCO_{3st}$ (*cf* Calculations p 17) Thus the $P_{a_{CO_2}}$ reached a peak value in the second and third minutes of exercise, and then declined slowly, as opposed to $[H^+]_a$ which remained relatively constant after the first 2—3 min of exercise Although in the 5th and 6th min of exercise $P_{a_{CO_2}}$ showed a significant increase of about 3 mm Hg *in all three conditions (Table IV), there was a rather wide scatter of the* individual increments (Fig 7) No relation could be found between the magnitude of increase in $P_{a_{CO_2}}$ and the working capacity

In recovery $P_{a_{CO_2}}$ had a time course similar to $[H^+]$ However, due to the diminished buffering capacity, $P_{a_{CO_2}}$ fell below the pre exercise level although $[H^+]_a$ remained elevated

Analysis of Ventilatory Changes at the Start and Cessation of Exercise

For the first 90 seconds of both exercise and recovery a breath by breath determination of the respiratory minute volume was made from the continuous recordings of the tidal volumes From estimates of individual values, the group *means of the time averages for consecutive* 6 sec periods were calculated for the first 42 sec For the remaining time periods the respiratory minute volume was calculated *as time averages over 15 sec periods The group means have* been plotted in Fig 8

On Effects Immediately at the onset of exercise *the respiration was in*creased, and even the very first breath was invariably augmented (*cf* Fig 6, p 29) This very rapid rise of ventilation in the initial stage nearly doubled the ventilation during the first 6 sec of exercise The initial rise was larger and more distinct in HB and LB (Fig 8, *B* and *C*) In all three conditions the ventilatory increase in the first half minute of exercise corresponded to 40—60 % of the total ventilatory increase in the 5th and 6th min of exercise

'Off' Effects Immediately at the cessation of exercise there was a sudden decrease in ventilation in all three conditions The most marked decrease was seen in NB (*A*) Within the first 2—3 breaths *the increase in respiratory* minute volume above the resting level was reduced to some *two thirds of the* increment in the 5th and 6th min of exercise

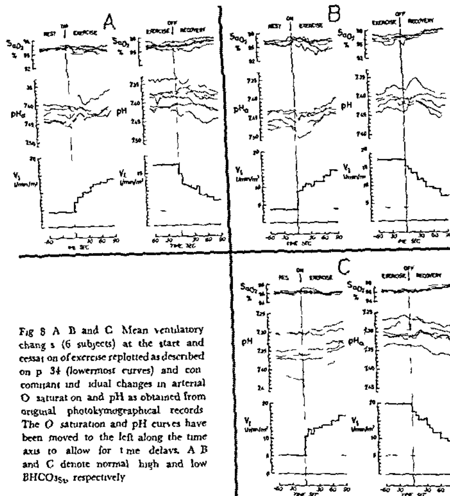


Fig 8 A B and C Mean ventilatory chang s (6 subjects) at the start and cessat on of exercise replotted as described on p 34 (lowermost curves) and con comitant ind idual changes in arterial O saturat on and pH as obtained from original photokymographical records The O saturation and pH curves have been moved to the left along the time axis to allow for t me delays A B and C denote normal high and low $BHCO_{3_{SB}}$ respectively

After the initial fall there was a stage of a secondary more gradual decre ment of ventilation the beginning of which took place some 20 sec afte cessation of exercise

pH and S_0 For comparison the original S_0 and pH recordings hav been replotted on the same coordinates in Fig 8 The recordings were move to the left along the time axis to allow for inherent time delays

During the first 20 seconds of exercise and recovery the pH_a recordin showed in general no consistent changes After about 30 seconds all curv changed in the same direction during exercise in an acid direction in recove towards the pre-exercise levels

Some subjects exhibited a peculiar variation in pH_a at the onset of exercise, with an alkalotic drop immediately after onset of exercise followed by a transient acid wave However, the mean level of the individual tracings during the first 30 sec was *unchanged or showed a slightly acid shift* The alkalotic drop was especially pronounced in subject JF (lowest pH tracings in Fig 8, *B* and *C*)

The individual scatter in $S_{a_{O}}$ was very small The constancy of the individual tracings during the first half minute of exercise and recovery was striking in all three conditions The slight lowering of $S_{a_{O}}$ at the end of the first minute of exercise (p 28) can be especially seen in *B* and *C*

In summary, the analysis of the respiratory changes at the onset and at the cessation of exercise revealed rapid and large changes of V_I in the initial stages of both exercise and recovery The magnitude of the changes was considerable, amounting to about 50 per cent of the total ventilatory increment during exercise No consistent changes occurred in the chemical composition of the arterial blood during these initial changes of ventilation

Increments in V, Arterial [H+] and P_{CO_2} *during* CO_2 Inhalation and during Exercise and Recovery

In the preceding two sections it was shown that pulmonary ventilation during moderate, constant load exercise exhibited an initial rise, followed by a slower *secondary increase* until a plateau was attained During recovery, an initial fall occurred, followed by a slower secondary decrease Furthermore, it was shown that the *initial ventilatory changes at the transitions from rest to exercise* and from exercise to rest were quite rapid if analyzed on a breath by breath basis By combining the V curves of Fig 7 and 8 one arrives at the typical time course of V showing short lasting plateaus during the very first stages of exercise and recovery before progressing as shown in Fig 7 (The result of this operation is schematically represented by the upper curve in Fig 10 from which the initial fast and secondary slow changes in V during the periods of exercise and recovery are apparent)

A close study of the individual response pattern of the arterial pH during the period of exercise rev*ealed a striking similarity to that of* V during what is here referred to as the stage of secondary increase in V (*cf* Fig 10) Further more, the intra individual relation between the increments in V and arterial [H+] during this stage seemed to match the individual $\Delta V_I/\Delta$ [H] slope obtained during CO_2 inhalation at rest (*cf* upper graphs in Fig 5) It there fore *seemed* worthwhile to investigate these relations for the entire group Fig 9 A shows the mean increments in V as observed (1) in the stage of secondary increase *during* exercise (ΔV in Fig 10) and (2) during CO_2 inha

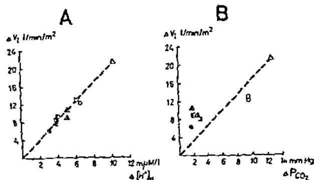


Fig 9 Mean increments in ventilation plotted against those in arterial hydrogen ion concentration (A) and carbon dioxide tension (B) as observed under the following conditions

1 during CO breathing (open symbols) or shifting from 2.8 CO to 6.3% CO_2 in air (cf Table III absolute values represent time-averages over the 9th and 10th min on each gas mixture)

2 during the stage of secondary increase in exercise (symbols filled on right side)

3 during early recovery (symbols filled on left side)

Circles – normal $BHCO_{3_{St}}$ squares = high $BHCO_{3_{St}}$ triangles = low $BHCO_{3_{St}}$

lation on changing from one CO_2 fraction to another both plotted against the concomitant increments in arterial [H+]

The relation between increments in V and arterial [H+] was also studied during recovery As soon as the fast initial fall of ventilation had subsided this relation (ΔV /Δ[H] in Fig 10) assumed a value that was close to the ΔV /Δ[H] slope during the stage of secondary increase in exercise and for CO_2 inhalation (cf above) That the mean increments in V and [H+] as studied above were well correlated is further demonstrated by the fact that the ΔV/Δ[H+] relationships were found to yield practically the same slope whether studied in the normal high or low $BHCO_{3}$ condition (circles squares and triangles in Fig 9) It can be seen from Fig 9 A that a change in the arterial [H] by one unit (1 mμM/l) corresponded to a ventilatory change of about 2 l/m² body surface

For comparison all the relations described above have been plotted with arterial P_{CO_2} substituted for arterial [H+] as the abscissa (Fig 9 B) The information contained in Fig 9 A and B indicates that in general, the ventilatory increments during exercise and recovery (ΔV and ΔV ') and CO_2 inhalation under normal as well as under high and low $BHCO_{3}$ conditions are better correlated with the concomitant increment in [H+] than with that of $P_{a_{CO_2}}$ Fig 9 also shows that the ΔV/ΔP$_{CO_2}$ slope was steeper during exercise and recovery than following inhalation of CO_2

Some subjects exhibited a peculiar variation in pH_a at the onset of exercise, with an alkalotic drop immediately after onset of exercise followed by a transient acid wave However, the mean level of the individual tracings during the first 30 sec was unchanged or showed a slightly acid shift The alkalotic drop was especially pronounced in subject JF (lowest pH tracings in Fig 8, *B* and *C*)

The individual scatter in S_{a_O} was very small The constancy of the individual tracings during the first half minute of exercise and recovery was striking in all three conditions The slight lowering of S_{a_O} at the end of the first minute of exercise (p 28) can be especially seen in *B* and *C*

In summary, the analysis of the respiratory changes at the *onset* and at the cessation of exercise revealed rapid and large changes of V_I in the initial stages of both exercise and recovery The magnitude of the changes was considerable, amounting to about 50 per cent of the *total ventilatory increment* during exercise No consistent changes occurred in the chemical composition of the arterial blood during these initial changes of ventilation

Increments in V, Arterial [H+] and P_{CO_2} during CO Inhalation and during Exercise and Recovery

In the preceding two sections it was shown that pulmonary ventilation during moderate constant load exercise exhibited an initial rise, followed by a slower secondary increase until a plateau was attained During recovery, an initial fall occurred, followed by a slower secondary decrease Furthermore, it was shown that the initial ventilatory changes at the transitions from rest to exercise and from exercise to rest were quite rapid if analyzed on a breath by breath basis By combining the V curves of Fig 7 and 8 one arrives at *the typical* time course of V showing short lasting plateaus during the very first stages *of* exercise and recovery before progressing as shown in Fig 7 (The result of this operation is schematically represented by the upper curve in Fig 10 from which the initial fast, and secondary slow changes in V during *the periods* of exercise and recovery are apparent)

A close study of the individual response pattern of the arterial pH during the period of exercise revealed a striking similarity *to that of V during what* is here referred to as the stage of secondary increase in V (*cf* Fig 10) Further more, the intra individual relation between the increments in V and arterial [H+] during this stage seemed to *match* the individual $\Delta V_I/\Delta [H^+]_a$ slope obtained during CO inhalation at rest (*cf* upper graphs in Fig 5) *It there* fore seemed worthwhile to investigate these relations for the entire group Fig 9 A shows the mean increments in V as observed (1) in the stage of secondary increase during *exercise* (ΔV in Fig 10) and (2) during CO_2 inha

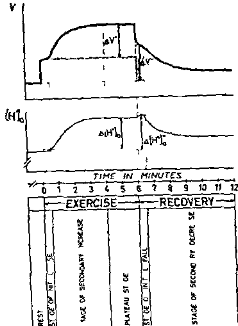


Fig 10 Basic form of the time-course of ventilation (V) during exercise and recovery as obtained in the present investigation (*heavy line*) Also shown schematically is the time-course of arterial [H] (from arterial pH recordings) The nomenclature used for the various stages in exercise and recovery refer solely to the observed changes in V

ΔV represents the increment of V from its mean value during the stage of initial rise to that in the plateau stage ΔV represents the difference between the mean value of V during the stage of initial fall in recovery and its resting level

Note that the ΔV/Δ[H] ratios in the plateau stage were the same as in the stage of initial fall in recovery

significant changes in these factors do not occur until after the lapse of about 20—30 sec

The observation that the ventilation at the onset of exercise increases independently of known arterial stimuli confirms the conclusions of KROGH and LINDHARD (1913b) ASMUSSEN and NIELSEN (1948) and DEJOURS (1959) In this connection it should be noted that the dynamic changes in the arterial P_{CO_2} as far as the first 20—30 sec are concerned, have in the past always been estimated indirectly from the measured changes in end tidal or end expiratory P_{CO_2} The present investigation has demonstrated however that end tidal P_{CO_2} is only to a limited extent representative of the arterial P_{CO_2} during the various phases of moderate exercise and subsequent recovery In effect a considerable rise in the end tidal P_{CO_2} could be observed at the transition from rest to exercise although the arterial P_{CO_2} still remained unchanged (*cf* p 20) Similarly the transition from exercise to rest may show a marked fall in the end tidal P_{CO_2} in the face of an essentially unchanged arterial P_{CO_2}

From these observations it can then be concluded that the actual changes in the arterial P_{CO_2} are even less than has earlier been surmised on the basis

VII DISCUSSION

Some of the more important *conclusions* that can be drawn from the results of the present investigation rest upon the comparison of ventilatory changes with rather small changes in arterial [H+] and P_{CO_2}. The validity of the comparisons will accordingly be dependent on the accuracy achieved in the determination of these changes. Even minute errors in the determination of the changes observed in arterial [H+] or P_{CO_2} during exercise will appreciably influence the relations described in the last chapter of the results.

It was therefore considered essential that the intraindividual changes in these parameters should be determined with great accuracy. This requirement was fulfilled by the use of continuous recording techniques for arterial *pH and* S_{O_2}, which have been in use for some years in this laboratory. The drift of the recording system was negligible, and the scatter inherent in spot sampling techniques was thus avoided.

In the following discussion the results obtained will be dealt with as they occur in the different stages of the ventilatory response to exercise. The characteristic features of this ventilatory response, which have been schematically illustrated in Fig. 10 (upper tracing), consist of a rapid, almost instantaneous rise of ventilation at the onset of exercise, followed by a more gradual increase to an apparently steady state (ASMUSSEN and NIELSEN 1948, DEJOURS 1959). At the cessation of exercise there is a sharp drop in ventilation *followed* by a gradual decrement. In the following discussion the nomenclature indicated in the figure will be used in referring to the different stages of the ventilatory response to exercise.

The Transition from Rest to Exercise and from Exercise to Rest

The results of the present investigation clearly demonstrate that the rapid changes in pulmonary ventilation observed at the onset and at the end of the exercise period *occurred before* any chemical changes in the arterial blood (as recorded or calculated) could have reached central or peripheral chemoreceptors. From the present data, obtained by the use of continuous recording techniques, it seems justified to state that physiologically

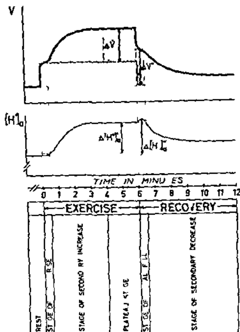


Fig. 10 Basic form of the time-course of ventilation (V) during exercise and recovery as obtained in the present investigation (*heavy line*). Also shown schematically is the time course of arterial $[H^+]$ (from arterial pH recordings). The nomenclature used for the various stages in exercise and recovery refer solely to the observed changes in V.

ΔV represents the increment of V from its mean value during the stage of initial rise to that in the plateau stage. $\Delta V'$ represents the difference between the mean value of V during the stage of initial fall in recovery and its resting level.

Note that the $\Delta V/\Delta[H^+]$ ratios in the plateau stage were the same as in the stage of initial fall in recovery.

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From these observations it can then be concluded that the actual changes in the arterial P_{CO_2} are even less than has earlier been surmised on the basis

of end tidal P_{CO_2} measurements Hence, the independence of the initial changes in ventilation with respect to the arterial P_{CO_2} at the transition from rest to exercise and from exercise to rest will be fully revealed only if the latter variable is measured directly

The relatively long lasting constancy of the arterial gas tensions would imply that the overall ventilation/perfusion ratio of the lung remained constant in early exercise, in spite of the rapid rise in ventilation This seems to indicate that the rapid ventilatory increase was accompanied by a similar rapid increase of blood flow from the very beginning of exercise Even a minor phase displacement between the acceleration of circulation and of ventilation would have caused major changes in the composition of the arterial blood

It thus seems as if the rapid increases in both the circulation and ventilation at the onset of exercise are initiated by neural mechanisms Because of the close association in time of circulatory and ventilatory changes it is tempting to postulate a common link responsible for the acceleration of both systems, either at the receptor sites, or within the central nervous system Such a hypothesis does not seem to be incompatible with current knowledge of circulatory adaption to exercise (*cf* ASMUSSEN and NIELSEN 1955)

It has recently been suggested by ARMSTRONG *et al* (1961) as well as by RILEY (1963) that pulmonary arterial chemoreflexes sensitive to changes in the composition of the mixed venous blood might contribute to the exercise hyperpnea It appears unlikely, however, that such chemoreflexes should be able to cause an increased ventilatory response within the first seconds of muscular exercise and thus before mixed venous blood of altered composition reaches the pulmonary artery (*cf* DEJOURS, MITHOEFER and TEILLAC 1955 b)

The neural stimuli supposed to increase ventilation may be either central or proprioceptive, and evidence has been presented that both types of stimuli may be acting (for review see ASMUSSEN and NIELSEN 1948, and DEJOURS 1959)

Apart from proprioceptive reflexes, other reflex mechanisms may be involved in the initial stage of ventilatory increase Hypotension may increase the reflexogenic stimulation to breathing from peripheral arterial baro and chemoreceptors (*cf* HEYMANS and NEIL 1958) A contribution of hypotension to the exercise hyperpnea has been suggested by KRAMER and GAUER (1941) An arterial pressure drop at the onset of exercise has been demonstrated in man by HOLMGREN (1956) who reported that the fall began some 3—4 sec after the onset of exercise and lasted for about 15 sec The fall varied between 0—40 mm Hg, and in most cases the reduction exceeded 10 mm Hg The findings of HOLMGREN have been confirmed in this laboratory (BARR *et al* 1963) In view of the fact that the initial rise in ventilation reached its peak before this hypotensive drop occurred it is questionable however if such an influence from peripheral receptors can contribute significantly to the initial rise of ventilation in man

The Role of Chemoreflexes during Exercise and Recovery

During moderate exercise there exists a close adjustment of the pulmonary ventilation to the metabolic needs of the body, so that the ventilatory equivalent (V_E/V_{O_2}) remains constant As pointed out by GRODINS (1950) an adequate theory of respiratory control must account for this observed relationship between ventilation and oxygen consumption in addition to qualitatively identifying the stimuli and pathways concerned In view of this precise adjustment of ventilation to the oxygen needs of the exercising body efforts have logically been made to detect a possible contribution of a hypoxic drive during exercise

The continuous recording of the oxygen level in the arterial blood revealed a surprising constancy of the arterial oxygen saturation during the course of exercise and recovery The metabolic alkalosis or acidosis present in conditions HB and LB, respectively did not change the oxygen saturation significantly although the arterial oxygen tension values were about 4 mm Hg lower in HB and about 8 mm Hg higher in LB than in NB Otherwise the pattern of the changes in oxygen tension was similar in the three conditions (*cf* Fig 7) The differences in the oxygen tensions in the three conditions and in the course of exercise were balanced by the changes in the arterial blood reaction so that the oxygen saturation remained almost unchanged The statement by GRODINS cited above on the constancy of the ventilatory equivalent may apparently be extended to include a notable constancy of the oxygen saturation of the arterial blood as being another factor that must be accounted for in any theory concerning the regulation of ventilation during exercise

The observation that the arterial oxygen tension was somewhat increased rather than decreased in the plateau stage of exercise agrees with recent results obtained by ASMUSSEN and NIELSEN (1958) LAMBERTSEN *et al* (1959) MCILROY and HOLMGREN (1961) and BARR *et al* (1963) Earlier observations of a fall in P_{O_2}, especially during heavy exercise (SUSKIND *et al* 1950 HOLMGREN and LINDERHOLM 1958) might to some extent be explained by the fact that the oxygen tensions were determined at 37 C instead of at the actual body temperature Another important factor when determining changes in P_{O_2} is the choice of control level In the sitting or standing position at rest, P_{O_2} is a few mm Hg lower than in the supine position due to the effect of posture on the pulmonary gas exchange (*cf* ULMER and REICHEL 1961 BJURSTEDT *et al* 1962) Thus had the control values been obtained with the subjects in the supine position the increase in oxygen tension during exercise would probably have become about half as great as that observed

The time course of the arterial P_{O_2} during moderate exercise has been investigated by SUSKIND *et al* (1950) These authors found the oxygen tension to be significantly lowered in the second and third minutes of exercise but

of end tidal P_{CO_2} measurements Hence, the independence of the initial changes in ventilation with respect to the arterial P_{CO_2} at the transition from rest to exercise and from exercise to rest will be fully revealed only if the latter variable is measured directly

The relatively long lasting constancy of the arterial gas tensions would imply that the overall ventilation/perfusion ratio of the lung remained constant in early exercise, in spite of the rapid rise in ventilation This seems to indicate that the rapid ventilatory increase was accompanied by a similar rapid increase of blood flow from the very beginning of exercise Even a minor phase displacement between the acceleration of circulation and of ventilation would have caused major changes in the composition of the arterial blood

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(EULER, LILJESTRAND and ZOTTERMAN 1939) EULER and LILJESTRAND (1946) have also demonstrated a diminution in the ventilatory response to electrically induced exercise in animals after denervation of the chemoreceptors

Interrelationships of Ventilation and Arterial [H⁺] and P_{CO_2} during Exercise and Recovery

The total exercise hyperpnea vs hypercapnic hyperpnea In condition NB the hyperpnea induced by inhalation of 6 3 % CO_2 in air was of approximately the same magnitude as the hyperpnea in the steady state of exercise However the *increments in arterial [H⁺] and P_{CO_2} during exercise* were only about one half and one third respectively of those induced by CO_2 inhalation It may thus be concluded that the observed changes in *the H^+ CO_2 complex* were much too small to account for the hyperpnea of exercise

In condition LB the inhalation of CO_2 caused a more marked hyperpnea The corresponding changes in P_{CO_2} and especially in [H⁺] were however also much larger so that the relation between ventilatory increase and increase in [H] remained unchanged The increments in ventilation and [H⁺] during exercise were somewhat larger than in condition NB whereas the *increase in P_{CO_2} was approximately the same These observations indicate* that during exercise there seems to be a relation between the ventilatory *increase* and the increase of [H⁺]

The stage of secondary increase in $\dot{V}$ When the ventilatory increments during the stage of secondary increase in ventilation were plotted against the concomitant increments in [H⁺] ($\Delta \dot{V}/\Delta$[H⁺] in Fig 10 p 39) it was found that in all three conditions these points fell close to the $\Delta \dot{V}/\Delta$[H⁺] response lines obtained during CO_2 inhalation *The slopes of these CO_2 response lines* were almost identical in the three conditions (Fig 9 A p 37) When the same plots were made for the relation between $\Delta \dot{V}$ and ΔP_{CO_2} *the points fell to* the left of the CO_2 response lines (Fig 9 *B*)

The remarkably good correlation between changes in ventilation and changes in arterial [H⁺] in all the conditions studied does not seem to have been described previously From this observation it may be justified to conclude that the response of the respiratory system to changes in arterial [H⁺] is the same whether these changes are induced by exercise or by CO_2 inhalation It also appears that the observed relations between the ventilatory increase in the secondary stage of exercise hyperpnea and the concomitant increments in [H] give support *to those hypotheses which anticipate an interaction of* humoral and neural respiratory drives in the generation of the exercise hyperpnea (*cf* DEJOURS 1959 LAMBERTSEN 1961 and CUNNINGHAM 1963)

otherwise no significant changes were observed in the 4 min of exercise, and 3 min of recovery studied In the present investigation a slight decrease was found in the second halfminute of exercise, indicating that V_{O_2} increased somewhat faster than V_A This transient lowering of the oxygen tension appears to be too small to constitute a significant causative factor of the exercise hyperpnea GALDSTON and WOLLACK (1947) found that the P_{aO_2} in the first minute of recovery was about 13 mm Hg higher than in the resting supine position A similar increase was found in the present investigation

In an earlier investigation from this laboratory (BARR *et al* 1963) the time courses of arterial pH and S_{O_2} were investigated during light and moderate exercise without measurement of V_E The time courses of the two variables were found to be almost identical with those obtained in the present investigation This indicates that the constancy of S_{aO_2} and the increase in P_{aO_2} were not caused by any interference with respiration due to increased external breathing resistance In a study of the changes in cardiac output during exercise, DONALD, BISHOP and WADE (1954) also determined the time courses of the changes in arterial S_{O_2} As judged from their graphs, S_{O_2} remained approximately unchanged during the course of exercise and recovery

The observation that the arterial P_{O_2} remains unchanged or even shows a small increase during moderate exercise does not necessarily imply an unchanged or decreased hypoxic chemoreflex drive The hypoxic drive during exercise has been indirectly studied in man in experiments performed at different degrees of hyperoxia Numerous such investigations (*e g* ASMUSSEN and NIELSEN 1946, 1958 LUNDIN and STROM 1947 BANNISTER and CUNNINGHAM 1954, LAMBERTSEN *et al* 1959 PERRET 1960) have shown that oxygen inhalation diminishes the ventilatory response to exercise, the more so the heavier the work load Oxygen administration also lowers the lactic acid level during exercise Conversely, during moderate hypoxia ventilation is increased in proportion to the increased lactacidemia (*cf* ASMUSSEN and NIELSEN 1946) However, the effect of high oxygen pressure is not exclusively related to improved oxygenation in the working muscle Judging from the single breath O_2 test technique, described by DEJOURS *et al* (1957a 1958) the hypoxic chemoreflex drive contributes to some 10—15 % of the exercise hyperpnea during air breathing at sea level, and even more during hypoxia (DEJOURS 1959, MILIC EMILI RAYNAUD and DEJOURS 1960) It appears that the single breath O_2 test provides quantitative information as to the hypoxic chemo reflex part of the ventilatory drive, since the reduction of ventilation following an ' O_2 test ' occurs within one circulation time Actually the ventilatory reduction at rest is maximal about 7 sec after the pulmonary capillary P_{O_2} has attained its maximal value (DEJOURS and MATELL 1962, unpublished observations) The demonstration of a certain hypoxic drive in man during air breathing at sea level agrees with observations in animal experiments of a tonic discharge from the chemoreceptors even at normal oxygen pressures

after the first half minute of exercise has a constant relation to the increase in [H+] The black areas which constitute the remaining part of the total ventilatory increase would thus correspond to the non arterial contributions to the respiratory drive and are believed to include mainly the neural respiratory drives The fact that these black areas have a rectangular shape gives some support to the view that the neural respiratory drives are acting throughout the exercise period as has been proposed by DEJOURS (1959) This does not imply however that the neurogenic drive remained constant Such factors as e g body temperature and adrenaline levels may also contribute to the exercise hyperpnea Since the temperature rise observed in the present investigation amounted only to some tenths of a degree it is believed that the rise in temperature contributed but little to the exercise hyperpnea

The hyperpnea of recovery After the initial rapid fall in ventilation the ratio $\Delta V / \Delta[H^+]$ in early recovery (*cf* Fig 10 p 39) had the same value as $\Delta V / \Delta[H^+]$ during exercise Later in recovery ventilation decreased faster than the hydrogen ion concentration This may to some part be explained by a probable decrease in hypoxic stimulation during recovery but it also seems that other unknown factors contributed to this ventilatory decrement

Concluding remarks The present investigation has shown that irrespective of the buffering capacity of the blood there was the same relation between changes in [H+] and ventilation both during CO_2 inhalation and during the secondary stages of ventilatory changes in response to exercise No such constant relation was found between changes in P_{CO_2} and ventilation This does not necessarily imply however that H^+ is the unique respiratory stimulus A differentiation between the physiological action of [H+] and P_{CO_2} is rendered difficult by the fact that changes in P_{CO_2} are accompanied by concurrent alterations of [H] if $BHCO_{3s}$ remains constant

In this connection it should be emphasized that the observed correlation between ventilation and [H] seems to be valid only in acute experiments This conclusion is based on the observation that the longlasting acidemia induced by ammonium chloride ingestion was associated with only a slight increase of the resting ventilation which is in agreement with earlier observations of e g NIELSEN (1936)

Available data seem to indicate that a number of respiratory stimuli contribute to the hyperpnea of exercise The coarse adjustment of ventilation appears to be of neural origin and related to the rhythm strength and degree of engagement of the musculo-skeletal system Humoral stimuli seem to be responsible for the fine and precise adjustment of ventilation to the metabolism apparently in a similar way as in the resting condition

In this investigation the two main groups of stimuli seemed to be responsible for about equal parts of the hyperpnea during exercise If the same relation exists for other loads and types of work remains to be investigated

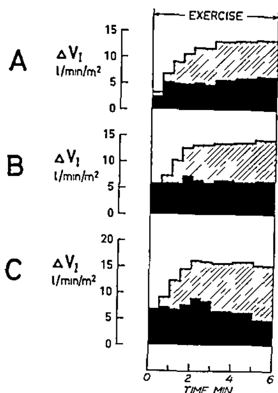


Fig 11 Schematic representation of calculated humoral and non humoral components in exercise hyperpnea

The boundaries of any one graph represent the time course of the ventilatory increase (above pre exercise level) during the period of exercise (cf Fig 7) The hatched area has been obtained from the actual increase in $[H^+]$ and the $\Delta V/\Delta[H^+]_a$ ratio observed during CO_2 inhalation at rest (chemically accountable ventilation) The black area represents the remaining component of respiratory stimulation

The graphs are based on data from continuous recordings which were reduced into group means of individual time averages determined over the first six 30 sec periods and the ensuing three 60 sec periods during exercise (625 kpm/min for 6 min)

A = Normal $BHCO_{3St}$ (n = 5)
B = High $BHCO_{3St}$ (n = 5)
C = Low $BHCO_{3St}$ (n = 6)

The plateau stage of exercise As shown in Fig 7 (p 30) the ventilation and arterial $[H^+]$ remained relatively stable in the 5th and 6th min of exercise The magnitude of the increase in $[H^+]_a$ agrees with earlier observations (LAMBERTSEN *et al* 1959, BARR *et al* 1963) The rather inconsistent pattern of the P_{aCO_2} is also in agreement with findings reported in the literature (*cf* p 8)

The share of $[H^+]_a$ *in the exercise hyperpnea* The present observation of an essentially unaltered response of the respiratory system to changes in $[H^+]_a$ warrants a tentative analysis of the average share of $[H^+]_a$ in the exercise hyperpnea This has been done by multiplying the observed increments in $[H^+]$ by the value of $\Delta V/\Delta\ [H^+]$ obtained during CO_2 breathing In Fig 11 these products are indicated by hatched areas, which thus represent the share in the total ventilatory increase induced by increments in $[H^+]$ Admittedly, objections may be made against this way of processing the data *e g* because (a) the time necessary for establishing equilibrium between the gases in the blood and the chemosensitive areas is not known and (b) a possible additive action of P_{CO_2} as an independent respiratory stimulus has not been accounted for However the diagram is not intended to be more than an illustration of the possible relation between ventilation and $[H^+]$ obtained in this investigation

The diagram would then illustrate that the ventilatory increase appearing

after the first half minute of exercise has a constant relation to the increase in $[H^+]$ The black areas which constitute the remaining part of the total ventilatory increase would thus correspond to the non arterial contributions to the respiratory drive and are believed to include mainly the neural respiratory drives The fact that these black areas have a rectangular shape gives some support to the view that the neural respiratory drives are acting throughout the exercise period as has been proposed by DEJOURS (1959) This does not imply however that the neurogenic drive remained constant Such factors as *e g* body temperature and adrenaline levels may also contribute to the exercise hyperpnea Since the temperature rise observed in the present investigation amounted only to some tenths of a degree it is believed that the rise in temperature contributed but little to the exercise hyperpnea

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In this connection it should be emphasized that the observed correlation between ventilation and $[H^+]$ seems to be valid only in acute experiments This conclusion is based on the observation that the longlasting acidemia induced by ammonium chloride ingestion was associated with only a slight increase of the resting ventilation which is in agreement with earlier observations of *e g* NIELSEN (1936)

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In this investigation the two main groups of stimuli seemed to be responsible for about equal parts of the hyperpnea during exercise If the same relation exists for other loads and types of work remains to be investigated

VIII SUMMARY

In an attempt to assess quantitatively the role of possible chemical stimuli in the hyperpnea of exercise, the time courses of ventilation, arterial O_2 saturation and pH (and concomitant calculated changes in arterial P_{O_2} and P_{CO_2}) were studied in 7 healthy male subjects during moderate, constant load exercise (625 kpm/min for 6 min) and ensuing recovery (6 min), both under normal and under high and low $BHCO_3$, the two last mentioned conditions being induced by ingestion of ammonium chloride and sodium bicarbonate, respectively

1 *Ventilatory and blood chemical responses* At the onset of exercise ventilation increased in a stepwise fashion, and after some 20—30 sec showed a secondary increase to attain a plateau after about 3 min of exercise At the cessation of exercise ventilation first fell steeply and after 20—30 sec showed a secondary, more gradual decrease towards its pre exercise level

The arterial O_2 saturation remained remarkably constant throughout the period of exercise, *whereas the O_2 tension, after a transient drop increased* by about 5 mm Hg In recovery, the O_2 saturation increased by about 1 %, and the O_2 tension by about 15 mm Hg during the first 2 min and then declined

Arterial $[H^+]$ and P_{CO_2} remained essentially constant for the first 20—30 sec following the onset and cessation of exercise During the remainder of the period of exercise arterial $[H^+]$ increased gradually to reach a plateau after 3—4 min The arterial P_{CO_2} (mean for the group) was elevated at the end of exercise, however some individuals showed a decrease below resting levels

During the remainder of the period of recovery arterial $[H^+]$ showed a gradual decrease towards pre exercise levels The arterial P_{CO_2} (mean for the group) gradually fell below the pre exercise level

The observation that the initial rise and fall of ventilation in exercise and recovery, respectively occurred without significant changes in arterial O_2, H^+ or CO_2 levels, suggests that these fast components of exercise hyperpnea are controlled by other factors

2 *Assessment of H^+ and CO_2 stimuli in exercise hyperpnea* On determining individual and mean responses to CO_2 inhalation at rest and analysing these responses in terms of slopes ($\Delta V/\Delta[H^+]$ and $\Delta V/\Delta P_{CO_2}$) the mean $\Delta V/\Delta[H^+]_a$ slopes during the *secondary increase in exercise hyperpnea* and during the first half minute of *recovery* (subtracting the initial decrement in ventilation) were found to coincide with the $\Delta V/\Delta[H^+]$ slope obtained during CO_2 inhalation (2 l/m^2 BSA/mμM/l)

The $\Delta V/\Delta[H^+]$ and $\Delta V/\Delta P_{CO_2}$ slopes as defined above were investigated under normal as well as under high and low $BHCO_{3st}$ In general the $\Delta V/\Delta[H^+]$ slopes were about the same in all three conditions whereas the $\Delta V/\Delta P_{CO_2}$ slopes were steeper in exercise and recovery than under CO_2 inhalation especially in the low $BHCO_{3st}$ condition

Evidence is presented that the slow ventilatory increase in the secondary stage of exercise hyperpnea is related to the increase in [H+]

3 *Other results* It was observed that during exercise the arterial to end tidal P_{CO} difference became reversed (mean — 3 1 mm Hg) Also during inhalation of 6 3 % CO_2 a small negative difference (mean—0 9 mm Hg) was found The mechanisms underlying these reversals of the P_{CO_2} difference have been discussed

Ingestion of ammonium chloride (2 5 meq/kg body weight for 3 days) lowered the $BHCO_{3st}$ by 9 5 mM/l and ingestion of equivalent amounts of sodium bicarbonate increased the $BHCO_{3st}$ by 3 4 mM/l the normal value being 26 0 mM/l The lowering of the $BHCO_{3st}$ caused by exercise was not markedly different in the three conditions No significant differences were observed between the three conditions as to oxygen uptake RQ, and heart rate at rest or during exercise

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von

BERNHARD TRIBUKAIT

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Aus der Klinisch Physiologischen Abteilung des Karolinska Sjukhuset
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XI Arterielle O Hb Sattigung und alveolarer CO und O Druck der Ratte bei Aufenthalt in 2000—7000 m Hohe Acta psysiol cand 1963 In Druck

XII Der Einflu s von Cobalt auf die Erythropoiese der Ratte Acta physiol scand 1963 In Druck

Die Arbeiten werden im Text mit den ent prechenden romi chen Ziffern referiert

Unter dem Begriff Erythropoiese können alle Prozesse die zur Bildung eines reifen Erythrozyten führen also Zellteilung und Zellreifung sowie Hb Synthese verstanden werden Untersuchungen der Erythropoiese beschäftigen sich entsprechend primär mit morphologisch biochemischen Problemen des sich bildenden Erythrozyten Einen seinem Wesen nach anderen Fragekomplex bildet die Regulation der Erythropoiese zu deren Verständnis eine funktionelle Betrachtungsweise notwendig ist Während alle Fragen der Erythropoiese prinzipiell an dem Entwicklungsablauf einer einzigen Stammzelle bis zum Erythrozyten studiert werden könnten wenn dieses möglich wäre geht es bei der Regulation der Erythropoiese darum warum gerade eine bestimmte Anzahl von Zellen gebildet wird und wie das erreicht wird

Sowohl die Biochemie der Zellentwicklung wie die Dynamik der Zellteilung sind das Ziel umfassender Untersuchungen und Diskussionen gewesen (THOPELL 1958 ALPEN und CRANMORE 1959 LAJTHA und OLIVER 1960) Fragen der Regulation der Erythropoiese sind wesentlich durch die Existenz erythropoiesestimulierender Substanzen — Erythropoietine — und deren Nachweis bestimmt worden Seit REISSMANN (1950) zeigen konnte dass bei parabiotischen Ratten Hypoxie des einen Partners eine erhöhte Knochenmarksaktivität des anderen Partners hervorruft sind eine Fülle von Arbeiten über Auftreten Wirkungsweise chemische Zusammensetzung und Metabolismus derartiger Substanzen erschienen (Zusammenfassende Übersichten GORDON 1959 LINSMAN und BETHELL 1960 Ciba Foundation Symposium on Haemopoiesis 1960 JACOBSON und DOYLE 1962) Der Nachweis erythropoietisch aktiver Substanzen gründet sich ganz überwiegend auf das Verhalten relativer Blutwerte (Hb und Erythrozytenkonzentration Hämatokrit) das Auftreten von Retikulozyten die Höhe der Eiseninkorporation in Erythrozyten oder die Knochenmarksaktivität Nur vereinzelt ist die gesamte Erythrozytenmenge bestimmt worden

Die Studien humoraler Übertragermechanismen sind für das Verständnis der Regulation der Erythropoiese von großer theoretischer und praktischer Bedeutung Sie zeigen wie die Erythropoiese aktiviert wird eventuell auch welche Organsysteme und andere Faktoren an diesem Aktivierungsprozess beteiligt sind Dabei mag es genügen den erythropoietischen Aktivitätszustand

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und vor allem kleinen Versuch tier sind jedoch nur schwer moglich Es i t weiterhin zu berucksichtigen dass Anderungen im Plasmaeisen piegel in der Verteilung und im Zustand des Eisens die Hohe der Eiseninkorporation beein flu sen konnen ohne dass die Erythropoie e elbst ent prechend beteiligt zu sein braucht (HODGSON et al 1957) Ein veranderter Abstrom von Retikulozyten aus dem Knochenmark ins Blut wie z B unter Hypoxie (SAATHOFF 1950 a MERTENS 1957) oder auch bei fastenden Ratten (MEYER THEWLIS und RUSCH 1940) kann ebenso wie eine Eiseninkorporation in zirkulierende Retikulozyten zu einer fal chen Einschatzung der Erythropoiese fuhren Da Fe en auch in nicht an Ham gebundener Form im Erythrozyten vorkommt (FALBE HANSEN und LOTHE 1962) sollte die spezifi che Aktivitat des Hb und nicht die der Ery throzyten geme en werden Schlie slich kann eine gesteigerte Erythropoiese durch eine vorausgehende Hamolyse ausgelo t werden GORDON und WEINTRAUB (1962) fanden beispiel weise bei etwa 25 % von Tieren die mit erythropoie tisch aktiven Urinproben behandelt waren hämolytische Reaktionen und empfehlen deshalb gleichzeitig Kontrollen des Hamatokrit

Fur die Beurteilung der Erythropoie e nach der Retikulozytenzahl im peri pheren Blut gelten im wesentlichen ent prechende Überlegungen wie fur die Eiseninkorporationsmethode Ein Vorteil ist dass beliebig oft von kleinen Blutmengen die Retikulozytenzahl bestimmt werden kann Ein Nachteil ist dass die Retikulozytenzahl des peripheren Blutes we entlich vom Verteilungs zustand zwischen Knochenmark und zirkulierendem Blut (normalerweise etwa 1 1 — SAATHOFF 1950 b FINCH 1959) abhängt und kurzfristig auf ver schiedene Reize hin (z B Reizung von Hirnpartien — BEER 1942 SEIP 1953 LINKE 1959 Injektion von Ascorbin aure oder Natriumbicarbonat — MEYER THEWLIS und RUSCH 1940 Rontgenbestrahlung — TRIBUKAIT nicht veroffent licht) ansteigen kann Aber auch langanhaltende Retikulozytenanderungen bei hohem O Druck und bei Cobaltzufuhr (Arbeit IX XII) brauchen nicht mit Anderungen des Gesamt Hb verbunden zu sein

Mes ungen der gesamten Erythrozytenmenge mit radioaktiv gezeichneten Erythrozyten ermoglichen zwar in vielen Fallen eine verlassliche Beurteilung der Erythropoiese Bei kleinen Tieren kann aber bereits bei einer Einzelbestim mung durch Blutentnahmen Injektion der Erythrozyten u ahnl die Erythro poiese so gestort werden dass eine Zweitbe timmung nicht mehr sinnvoll ist Unter uchungen am elben Tier sind aber vor allem dann be onders wesentlich wenn die Erythropoiese nur kurzfri tig oder geringfugig verändert wird Derartige geringe Abweichungen von der Norm las en sich sonst nur an einem gro seren Ver uch und Kontrollmaterial nachwei en Es mag in diesem Zu ammenhang von Interesse sein dass bei Durchsicht von etwa 200 Unter uchungen uber erythropoetisch aktive Sub tanzen die erschienen sind

nach den relativen Blutwerten, den Retikulozytenwerten oder der Eisen inkorporation zu beurteilen Sie lassen aber die Frage unbeantwortet, warum eine bestimmte Zellzahl gebildet wird

Die vorliegenden Untersuchungen bilden einen Versuch, Wege aufzuzeigen wie man zu einem besseren Verstandnis dieses zentralen Problems der Regulation der Erythropoiese gelangen kann Absolute Voraussetzung dafür ist eine quantitative Betrachtungsweise Wie wesentlich eine solche auch fur andere Fragen der Erythropoiese sein kann, soll gleichzeitig an einigen Beispielen gezeigt werden Fur sich betrachtet muss eine konstante Grosse z B die Hb Konzentration oder gesamte Hb Menge eines Organismus unter Normalbedingungen als gegeben hingenommen werden, wenn es nicht gelingt sie zu ihrer Funktion und den an sie gestellten funktionellen Anforderungen in Beziehung zu stellen Entsprechend wird hier auf den Zusammenhang zwischen dem Hb als O Transportmittel und der O_2 Versorgung des Gewebes eingegangen Damit lassen sich bereits auch die Grenzen dieser Arbeit erkennen Der O_2 Bedarf die Kapillarisation und Durchblutung des Gewebes zusammen mit dem O Gehalt und dem O_2 Druck des Blutes bilden ein derartig komplexes Geschehen dass eine vollstandige Analyse weit uber den Rahmen dieser Arbeit hinausgeht

I Methodik

Ein vollstandiges Bild vom Zustand der Erythropoiese gewinnt man aus der Kenntnis der gesamten Erythrozyten bzw Hb Menge und der per Zeiteinheit gebildeten und abgebauten Erythrozyten bzw Hb Menge Es ist aus praktischen Grunden schwierig diese drei Grossen gleichzeitig zu bestimmen Im allgemeinen hat man sich deshalb darauf beschrankt den Aktivitatszustand oder Anderungen im Aktivitatszustand der Erythropoiese festzustellen

Die Hb und Erythrozytenkonzentration des Blutes bzw der Hamatokrit sind wesentlich vom Verhalten des Plasmavolumens abhangig und bilden im allgemeinen deshalb nur im Zusammenhang mit einer dritten Grosse wie der Retikulozytenzahl oder der Hohe der Eiseninkorporation eine anwendbare Messgrosse der Erythropoiese

Die grosste Verbreitung vor allem im Zusammenhang mit der Prufung erythropoietisch aktiver Substanzen hat die Eiseninkorporationsmethode gefunden Dabei wird normalen Tieren oder Tieren mit herabgesetzter Erythropoiese infolge Hypophysektomie Nahrungsentzug Hypertransfusion mit Erythrozyten oder Rontgenbestrahlung nach Gabe der zu testenden Substanzen radioaktives Eisen (Fe^{59}) injiziert und dessen Auftreten in den Erythrozyten des Blutes nach 12—24 Stunden gemessen Der Vorteil der Fe Inkorporationsmethode ist deren einfache Handhabung Wiederholte Bestimmungen am selben

und vor allem kleinen Versuchstier sind jedoch nur schwer möglich. Es ist weiterhin zu berücksichtigen, dass Änderungen im Plasmaeisenspiegel, in der Verteilung und im Zustand des Eisens die Höhe der Eiseninkorporation beeinflussen können, ohne dass die Erythropoiese selbst entsprechend beteiligt zu sein braucht (HODGSON et al. 1957). Ein veränderter Abstrom von Retikulozyten aus dem Knochenmark ins Blut wie z. B. unter Hypoxie (SAATHOFF 1950 a, MERTENS 1957) oder auch bei fastenden Ratten (MEYER, THEWLIS und RUSCH 1940) kann ebenso wie eine Eiseninkorporation in zirkulierende Retikulozyten zu einer falschen Einschätzung der Erythropoiese führen. Da Eisen auch in nicht an Häm gebundener Form im Erythrozyten vorkommt (FALBE-HANSEN und LOTHE 1962), sollte die spezifische Aktivität des Hb und nicht die der Erythrozyten gemessen werden. Schliesslich kann eine gesteigerte Erythropoiese durch eine vorausgehende Hämolyse ausgelöst werden. GORDON und WEINTRAUB (1962) fanden beispielsweise bei etwa 25 % von Tieren, die mit erythropoietisch aktiven Urinproben behandelt waren, hämolytische Reaktionen und empfehlen deshalb gleichzeitig Kontrollen des Hämatokrit.

Für die Beurteilung der Erythropoiese nach der Retikulozytenzahl im peripheren Blut gelten im wesentlichen entsprechende Überlegungen wie für die Eiseninkorporationsmethode. Ein Vorteil ist, dass beliebig oft von kleinen Blutmengen die Retikulozytenzahl bestimmt werden kann. Ein Nachteil ist, dass die Retikulozytenzahl des peripheren Blutes wesentlich vom Verteilungszustand zwischen Knochenmark und zirkulierendem Blut (normalerweise etwa 1 : 1 — SAATHOFF 1950 b, FINCH 1959) abhängt und kurzfristig auf verschiedene Reize hin (z. B. Reizung von Hirnpartien — BEER 1942, SEIP 1953, LINKE 1959; Injektion von Ascorbinsäure oder Natriumbicarbonat — MEYER, THEWLIS und RUSCH 1940; Röntgenbestrahlung — TRIBUKAIT, nicht veröffentlicht) ansteigen kann. Aber auch langanhaltende Retikulozytenänderungen bei hohem O_2-Druck und bei Cobaltzufuhr (s. Arbeit IX, XIII) brauchen nicht mit Änderungen des Gesamt-Hb verbunden zu sein.

Messungen der *gesamten* Erythrozytenmenge mit radioaktiv gezeichneten Erythrozyten ermöglichen zwar in vielen Fällen eine verlässliche Beurteilung der Erythropoiese. Bei kleinen Tieren kann aber bereits bei einer Einzelbestimmung durch Blutentnahmen, Injektion der Erythrozyten u. ähnl. die Erythropoiese so gestört werden, dass eine Zweitbestimmung nicht mehr sinnvoll ist. Untersuchungen am selben Tier sind aber vor allem dann besonders wesentlich, wenn die Erythropoiese nur kurzfristig oder geringfügig verändert wird. Derartige geringe Abweichungen von der Norm lassen sich sonst nur an einem grösseren Versuchs- und Kontrollmaterial nachweisen. Es mag in diesem Zusammenhang von Interesse sein, dass bei Durchsicht von etwa 200 Untersuchungen über erythropoietisch aktive Substanzen, die erschienen sind

nach den relativen Blutwerten den Retikulozytenwerten oder der Eisen inkorporation zu beurteilen Sie lassen aber die Frage unbeantwortet warum eine bestimmte Zellzahl gebildet wird

Die vorliegenden Untersuchungen bilden einen Versuch Wege aufzuzeigen wie man zu einem besseren Verstandnis dieses zentralen Problems der Regulation der Erythropoiese gelangen kann Absolute Voraussetzung dafur ist eine quantitative Betrachtungsweise Wie wesentlich eine solche auch fur andere Fragen der Erythropoiese sein kann, soll gleichzeitig an einigen Beispielen gezeigt werden Fur sich betrachtet muss eine konstante Grosse, z B die Hb Konzentration oder gesamte Hb Menge eines Organismus unter Normalbedingungen als gegeben hingenommen werden, wenn es nicht gelingt sie zu ihrer Funktion und den an sie gestellten funktionellen Anforderungen in Beziehung zu stellen Entsprechend wird hier auf den Zusammenhang zwischen dem Hb als O_2 Transportmittel und der O_2 Versorgung des Gewebes eingegangen Damit lassen sich bereits auch die Grenzen dieser Arbeit erkennen Der O_2 Bedarf, die Kapillarisation und Durchblutung des Gewebes zusammen mit dem O_2-Gehalt und dem O_2 Druck des Blutes bilden ein derartig komplexes Geschehen, dass eine vollstandige Analyse weit uber den Rahmen dieser Arbeit hinausgeht

I Methodik

Ein vollstandiges Bild vom Zustand der Erythropoiese gewinnt man aus der Kenntnis der gesamten Erythrozyten bzw Hb Menge und der per Zeiteinheit gebildeten und abgebauten Erythrozyten bzw Hb Menge Es ist aus praktischen Grunden schwierig diese drei Grossen gleichzeitig zu bestimmen Im allgemeinen hat man sich deshalb darauf beschrankt den Aktivitatszustand oder Anderungen im Aktivitatszustand der Erythropoiese festzustellen

Die Hb und Erythrozytenkonzentration des Blutes bzw der Hamatokrit sind wesentlich vom Verhalten des Plasmavolumens abhangig und bilden im allgemeinen deshalb nur im Zusammenhang mit einer dritten Grosse wie der Retikulozytenzahl oder der Hohe der Eiseninkorporation eine anwendbare Messgrosse der Erythropoiese

Die grosste Verbreitung vor allem im Zusammenhang mit der Prufung erythropoietisch aktiver Substanzen hat die Eiseninkorporationsmethode gefunden Dabei wird normalen Tieren oder Tieren mit herabgesetzter Erythropoiese infolge Hypophysektomie Nahrungsentzug Hypertransfusion mit Erythrozyten oder Rontgenbestrahlung nach Gabe der zu testenden Substanzen radioaktives Eisen (Fe^{59}) injiziert und dessen Auftreten in den Erythrozyten des Blutes nach 12—24 Stunden gemessen Der Vorteil der Fe^{59} Inkorporationsmethode ist deren einfache Handhabung Wiederholte Bestimmungen am selben

das Gesamt Hb dieser Tiere vor und nach der Blutentnahme bestimmt (Arbeit I) Die gefundene Differenz im Gesamt Hb entspricht der abgenommenen Menge Hb

Eine zufriedenstellende Reproduzierbarkeit der Werte ist auch nach 2—3 jahrigem Gebrauch der Methode festzustellen Die Normalwerte von unterschiedlich schweren und unter gleichen Leben bedingungen gehaltenen Tieren eines Stammes die wahrend dieser Zeit gemessen und in Arbeit II zusammengestellt worden ind weisen eine relativ geringe Streuung um die berechnete Regressionslinie auf Daraus kann gleichzeitig auch auf eine zufriedenstellende Homogenitat des verwendeten Tiermaterials geschlossen werden

2 *Darstellung von Hamin aus kleinen Blutmengen*

Fur Bestimmungen der Erythrozytenlebenszeit und zur Feststellung eventueller hamolytischer Reaktionen erschien es wunschenswert, nach Injektion von radioaktivem Glycin de sen Aktivitat in der Haminkomponente des Hb eines Tieres verfolgen zu konnen Um bei den dazu notwendigen Blutentnahmen den Blutverlust moglich t gering zu halten wurde eine Mikromethode zur Darstellung von Hamin aus 0 1—0,2 ml Blut entwickelt (Arbeit V)

3 *Arterielle O_2Hb Sattigung und arterieller O_2 Druck der Ratte in Hypoxie*

Es war notwendig die unter verschiedenen Hypoxiebedingungen gefundenen Blutwerte der Ratte nicht nur zum O Druck der Einatmungsluft ondern auch zum arteriellen O_2-Druck und zur arteriellen O Hb Sattigung relatieren zu konnen Derartige Daten von der Ratte cheinen jedoch zu fehlen Die arterielle O Hb Sattigung vom arteriellen Blut hypoxischer Tiere wurde deshalb nach VAN SLYKE bestimmt (Arbeit XI) Da aus technischen Gründen der arterielle O Druck nicht direkt geme en werden konnte, wurde indirekt am nichtnarkotisierten Tier der alveolare O_2-Druck bestimmt (Arbeit XI) und mit Hilfe der alveolaren arteriellen O_2-Druckdifferenz der arterielle O Druck errechnet Diese Bestimmung methode beruht im Prinzip auf dem Verhalten der COHb und O Hb-Sattigung bei Atmen von CO in einem geschlos enen Atemsystem

4 *Sonstige Methoden Tiermaterial Statistik*

Die sonstigen verwendeten Methoden sind in den jeweiligen Arbeiten in denen auch auf einschlagige Literatur verwiesen i t beschrieben Als Versuchstiere dienten mannliche Ratten eines Stammes (Wi tar) In einigen Untersuchungen wurden auch Kragenratten (Hooded rats National In titute for Medical Research Mill Hill London) verwendet Stati tische Berechnungen

nachdem BERLIN et al 1949 eine fur Kleintiere geeignete Messmethode der Gesamterythrozytenmenge mit Hilfe radioaktiv gezeichneter Erythrozyten beschrieben haben, nur in etwa 5 % dieser Untersuchungen das Erythrozytenvolumen bestimmt worden ist

Fur quantitative Untersuchungen der Erythropoiese und deren Veranderungen erschien es wunschenswert, uber eine Methode zu verfügen mit der wiederholt vom selben Tier absolute Blutwerte gemessen werden konnen Darauf wird nachfolgend eingegangen

1 a) *Bestimmung des Gesamthamoglobin und Blutvolumen*

Die in Arbeit I beschriebene Methode stellt die Weiterentwicklung eines von GEMZELL und SJOSTRAND (1954) angegebenen Verfahrens zur Bestimmung des Gesamt Hb mit CO dar Dabei befindet sich das Tier solange in einem geschlossenen zirkulierendem Atemsystem, dem eine bekannte Menge CO zugefuhrt wird, bis sich ein Gleichgewicht zwischen den Partialdrucken CO und O_2 der Atemluft einerseits und der COHb und O_2Hb Sättigung andererseits eingestellt hat Die Partialdrucke O_2 und CO stehen zur O Hb und COHb Sattigung entsprechend der Haldaneschen Gleichung in folgendem Verhältnis $pO_2/pCO = K$ O Hb/COHb wobei K eine Gleichgewichtskonstante ist Aus der Analyse der Gase des Atemsystem der vom Tier aufgenommenen CO Menge und dieser Gleichgewichtskonstanten errechnet sich die COHb Sättigung bzw Gesamtmenge Hb Das Blutvolumen ergibt sich au dem Gesamt Hb und der Hb Konzentration

b) *Vergleichsweise Bestimmung des Gesamthamoglobin mit CO und Cr^{51} gezeichneten Erythrozyten*

Um festzustellen wie die mit CO bestimmten Werte des Gesamt Hb mit denen ubereinstimmen die mit einer »konventionellen« Methode erhalten werden sind in Arbeit III Gesamt Hb und Blutvolumen normaler, anamischer und polyzythamischer Ratten jeweils gleichzeitig mit CO und Cr^{51} gezeichneten Erythrozyten bestimmt worden Bei allen drei Gruppen wurden mit der Cr^{51} Methode durchschnittlich 10 % niedrigere Werte gefunden Als Ursache dieser Differenz konnen neben systematischen Fehlern bei der Bestimmung der CO Konzentrationen oder anderer Grossen die Bindung von CO an nichtzirkulierendes Hb und Myoglobin sowie eine nicht vollstandige Durchmischung der mit Cr^{51} gezeichneten Erythrozyten mit dem Blut des untersuchten Tieres diskutiert werden

c) *Weitere Prufungen der Methode*

Um die Messgenauigkeit der Methode auch hinsichtlich absoluter Werte zu uberprufen wurde Tieren etwa 1/3 der Gesamtblutmenge entnommen und

das Gesamt Hb dieser Tiere vor und nach der Blutentnahme bestimmt (Arbeit I) Die gefundene Differenz im Gesamt Hb entspricht der abgenommenen Menge Hb

Eine zufriedenstellende Reproduzierbarkeit der Werte ist auch nach 2—3 jahrigem Gebrauch der Methode festzustellen Die Normalwerte von unter chiedlich chweren und unter gleichen Leben bedingungen gehaltenen Tieren eines Stammes die wahrend dieser Zeit gemes en und in Arbeit II zusammen gestellt worden sind wei en eine relativ geringe Streuung um die berechnete Regressionslinie auf Daraus kann gleichzeitig auch auf eine zufriedenstellende Homogenität des verwendeten Tiermaterials geschlossen werden

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Es war notwendig die unter ver chiedenen Hypoxiebedingungen gefundenen Blutwerte der Ratte nicht nur zum O Druck der Einatmungsluft sondern auch zum arteriellen O-Druck und zur arteriellen O Hb Sattigung relatieren zu konnen Derartige Daten von der Ratte scheinen jedoch zu fehlen Die arterielle O Hb Sattigung vom arteriellen Blut hypoxischer Tiere wurde deshalb nach VAN SLYKE bestimmt (Arbeit XI) Da aus technischen Gründen der arterielle O Druck nicht direkt gemessen werden konnte wurde indirekt am nichtnarkotisierten Tier der alveolare O-Druck bestimmt (Arbeit XI) und mit Hilfe der alveolaren arteriellen O-Druckdifferenz der arterielle O-Druck errechnet Diese Bestimmungsmethode beruht im Prinzip auf dem Verhalten der COHb und O Hb Sattigung bei Atmen von CO in einem geschlo enen Atemsy tem

4 *Sonstige Methoden Tiermaterial Statistik*

Die sonstigen verwendeten Methoden sind in den jeweiligen Arbeiten in denen auch auf einschlagige Literatur verwiesen ist beschrieben Als Versuchstiere dienten mannliche Ratten eines Stammes (Wistar) In einigen Untersuchungen wurden auch Kragenratten (Hooded rats National Institute for Medical Research Mill Hill London) verwendet Stati ti che Berechnungen

wurden nach allgemein ublichen Methoden (SNEDECOR 1956) ausgefuhrt Als Streuungsmass wurde wenn nicht besonders anders angegeben der mittlere Fehler der Mittelwerte $\left(\frac{\sigma}{\sqrt{n}}\right)$ verwendet Der Methodenfehler ist als Streuung eines einzelnen Wertes bei Doppelbestimmungen angegeben $\left(\frac{\sigma d}{\sqrt{2}}\right)$ d = Differenz einer Doppelbestimmung)

Diskussion zu I

Die moglichen Schlussfolgerungen aus Untersuchungen die sich mit Zustandsanderungen der Erythropoiese beschaftigen sind in hohem Grade von der Art der verwendeten Messmethoden abhangig Fur gewisse Fragen beispielsweise der qualitativen Prufung erythropoietisch aktiver Substanzen mogen nicht oder halbquantitative Messverfahren der Erythropoiese genugen Geht es wie hier darum, die Wirkung unterschiedlicher Reizstarken (z B Hypoxie verschiedenen Grades) und Reizformen (z B Anamie Hypoxie) auf die Erythropoiese zu studieren, miteinander zu vergleichen und auf ihre funktionelle Bedeutung hin zu prufen sind quantitative Messverfahren unumganglich notwendig

Die verwendete CO Methode ermoglicht es bei einem relativ kleinen Versuchsfehler beliebig oft und ohne Blutentnahmen vom selben Tier das Gesamt Hb zu bestimmen und damit dessen Veranderungen zu verfolgen Vergleichsweise Messungen des Gesamt Hb mit CO und Cr^{51} gezeichneten Erythrozyten ergaben fur die CO Methode um etwa 10 % hohere Werte Myoglobin vermag nach den in Arbeit I ausgefuhrten Berechnungen etwa 1—2 % der zugefuhrten CO Menge aufzunehmen und scheint somit nicht als wesentliche Ursache dieser Differenz in Frage zu kommen Die gemessene Menge des im Knochenmark fixierten Hb vermag etwa 4 % des CO zu binden Hinzu kommt das Hb der fakultativ zirkulierenden Retikulozyten die beim normalen Tier in einer Menge entsprechend 2—3 % der zirkulierenden Blutmenge in den Depots des Knochenmark vorkommen Dieses im Gewebe fixierte Hb mag bei der Berechnung des Blutvolumens aus dem Gesamt Hb und der Hb Konzentration zu einer Uberschatzung des Blutvolumens fuhren

Die Berechnungen der COHb Sattigung aus den Partialdrucken CO und O im Atemsystem und einer Gleichgewichtskonstanten ergeben nur dann korrekte Werte wenn die Gleichgewichtskonstante unter allen Bedingungen gleich ist Die Gleichgewichtskonstante wurde *in vivo* aus den Partialdrucken CO und O_2 des Atemsystems und der COHb und O Hb Sattigung des Blutes unter der Annahme normaler Ventilationsverhaltnisse mit einem alveolaren CO Druck von 10 mm Hg bestimmt Steigt die alveolare Ventilation und damit

der alveolare O Druck und ist die Grösse des An tiegs nicht bekannt muss die Gleichgewichtskonstante neu be timmt werden Das ist bei Anwendung der Methode an Tieren deren Atmung infolge Hypoxie verändert worden war geschehen (s Arbeit VII VIII X) Es ist aber auch möglich aus der bei Normalverhaltnis en gefundenen Konstanten der COHb und O Hb Sattigung und dem CO Partialdruck den alveolaren O bzw CO Druck zu berechnen In Arbeit XI wurde auf die e Wei e der alveolare O und CO Druck höhen adaptierter Tiere be timmt

II Ergebnisse

Die Erythropoese kann experimentell auf folgende Wei e timuliert wer den 1 durch herabgesetzten arteriellen O-Gehalt (Anamie CO Vergiftung Methamoglobinamie) 2 verminderten arteriellen O Druck (Hypoxie) 3 Zufuhr von Cobalt Die Wirkung dieser drei Reizformen zusammen mit dem Einfluss hohen O Drucks und hohen O Gehalts des Blutes auf die Ery thropoiese sind das Ziel einiger quantitativer Analy en gewe en

Es ist zweckma sig bei der Untersuchung die er Stimuli von eventuell be kannten gemeinsamen Angriffspunkten die er Reize in das Regulationsge schehen der Erythropoiese auszugehen Cobalt ist als Reizform mit dem erniedrigten O-Druck bzw O Gehalt des Blutes nicht ohne weiteres ver gleichbar da nach den Untersuchungen von BUCCIERO und ORTEN (1949) Cobalt das O Bindungsvermogen des arteriellen Blutes etwa durch Methämo globinbildung nicht beeinflusst Demgegenuber i t fur den herabgesetzten arteriellen O Druck wie fur den verminderten O-Gehalt die beide auf den O Druck des Gewebes einwirken ein gemeinsamer Angriffspunkt naheliegend Dabei unterscheiden sich Anamie und Hypoxie voneinander hinsichtlich ihrer Wirkung auf den O Druck des Gewebes etwa folgendermassen Bei Anämie sinkt unter der Pas age des Blutes durch das Gewebe die O Hb Sattigung auf ein niedrigeres Niveau als normal wobei die rechtsverlagerte O Hb Dissoziationkurve die Freisetzung des O vom Blut begunstigt In Hypoxie ist neben der O Hb Sattigung der O Druck des Blutes von vorn herein nied riger und somit auch das O-Druckgefalle zum Gewebe geringer Der ery thropoiese timulierende Effekt von Hypoxie ollte bei vergleichbaren Be dingungen des arteriellen O-Gehalts entsprechend tarker sein als der von Anamie

Die Auffas ungen uber die Wirkung von Hypoxie und Anämie stimmen jedoch durchaus nicht mit diesem Bild uberein Er i t verschiedentlich betont worden (RUHENSTROTH BAUER 1959 STOHLMAN 1959) dass Anämie einen tarkeren Reiz auf die Erythropoiese ausubt als Hypoxie ALTLAND und PARKER

(1955) fanden bei der Schildkröte in Hypoxie bei einem O_2 Druck zwischen 59 und 23 mm Hg wodurch die O Hb Sättigung um etwa 50 % herabgesetzt wird (McCutcheon 1947), keine gesteigerte Erythropoiese, wozu jedoch Blutentnahmen führten Stohlman (1959) lehnt auf Grund dessen und der Befunde, dass bereits sehr geringfügige Blutverluste oder chronische Hamolysen ohne nachweislich herabgesetzte Hb Konzentration die Blutbildung stimulieren überhaupt den O Druck des Gewebes als einheitliche Ursache der Erythro poiesesteigerung ab Eine solche findet sich auch nicht, wenn man mit Barcroft (1927) annimmt, dass bei Hypoxie der absinkende arterielle O- Druck Ursache der gesteigerten Erythropoiese ist, da bei Anämie der arterielle O- Druck unverändert ist Im gleichen Sinne mag sprechen, dass die Lebens länge unter Anämie gebildeter Erythrozyten nach Berlin und Lotz (1951) stark verkürzt ist, unter Hypoxie nach Fryers und Berlin (1952) jedoch nicht oder nur unbedeutend von der Norm abweicht

Es muss jedoch hier berücksichtigt werden, dass sich die Vergleiche zwischen Hypoxie und Anämie beim Säuger im wesentlichen auf Retikulozyten werte Eiseninkorporation oder indirekte Daten stützen Vor allem unter Hypoxie verschiedener Stärke fehlen quantitative Studien der Änderungen der Erythropoiese praktisch gänzlich Da Hypoxie mit Blutvolumenänderungen z B initial durch einen Flüssigkeitsverlust, verbunden ist kann auch hier nicht aus der Hb Konzentration auf Änderungen der Erythropoiese geschlossen werden, was bei Anämie in gewissem Grade möglich ist Die genannten Bestimmungen der Erythrozytenlebenslänge unter Anämie halten ebenfalls nicht einer diesbezüglichen Kritik stand, da sie auf dem Verhalten der spezi fischen Aktivität radioaktiv *in vivo* gezeichneten Hb beruhen und nicht die quantitativen Änderungen des Hb berücksichtigen

Bei der erörterten widerspruchsvollen Situation und um Probleme der Regu lation der Erythropoiese auf einer sichereren Basis diskutieren zu können wurden im einzelnen folgende Untersuchungen ausgeführt Nach akuter Blutungsanämie wurde die Regenerationsgeschwindigkeit des Hb die täglich gebildete Hb Menge und das Blutvolumen (Arbeit IV) sowie die Lebenszeit unter diesen Bedingungen neugebildeter Erythrozyten (Arbeit VI) bestimmt Eingehender wurde der Einfluss anhaltenden niedrigen O Drucks entsprechend 1000—8000 m Höhe (Arbeit VII) und im Zusammenhang damit auch hohen O- Drucks bei Atmung von 30—100 % O in Meereshöhe (Arbeit IX) unter sucht Eine Spezialstudie gilt dem initialen Anstieg der O Kapazität des Blutes bei Hypoxie (Arbeit VIII) Fragen des Zusammenhangs zwischen der er höhten Kapillarisation des Gewebes und Änderungen des O Gehalts des arteriellen Blutes als Mittel auf den O Druck des Gewebes einzuwirken werden bei Untersuchungen der Readaptation des höhenpolyzythämischen

Organi mus an Meereshohe beruhrt (Arbeit X) Entwicklung und Art der Polyzythamie unter dem Einfluss von Cobalt unter chiedlicher Dosierung sind in Arbeit XII untersucht

1 ANÄMIE

a) *Anämie als Rei. der Erythropoiese*

Etwa am 3 Tag nach Entnahme 1/3 des Blutvolumens beginnt das Gesamt Hb zu steigen Nach 10 Tagen sind etwa 60 % nach 20 Tagen 100 % des Hb Verlustes er etzt Die taglich gebildete Hb Menge erreicht zwischen 3 und 4 Tag mit einem etwa verdoppelten Wert ein Maximum und fallt bis zum 23 Tag langsam auf das Ausgangsniveau ab Das Blutvolumen ist unverandert

b) *Lebens eit der nach akuter Blutung gebildeten Erythrozyten*

Nach Entnahme 1/3 des Blutvolumens wurde auf der Hohe der Hb Neubildung am 3 Tag Ratten etwa 10 μC C^{14} 2 Glycin injiziert und das Verhalten der Gesamtaktivitat des Hamin berechnet aus der pezifischen Aktivitat und der mit CO gleichzeitig be timmten Gesamtmenge Hämin verfolgt Die so gemessene Lebenslange der Erythrozyten von Anämietieren und Normaltieren war mit etwa 55 Tagen gleich

2 HYPOXIE UND HYPEROXIE

Eine seit den er ten experimentellen Untersuchungen PAUL BERT S (1878) uber die Wirkung von Hypoxie auf die Blutbildung vieldi kutierte aber nach wie vor ungeklarte Frage i t wo und wie der O in die Regulation der Erythropoiese eingreift und welche Bedeutung der arterielle O Druck und der arterielle O Gehalt in diesem Zusammenhang haben

BARCROFT (1927) gibt in einer bekannten Monographie uber Die Atmungsfunktion des Blutes dem verminderten arteriellen O-Druck vor dem ab inkenden O-Gehalt klar den Vorrang »Und zwar ist die Begrundung da s die Hamoglobinzunahme o gross sein kann dass sie eine wirkliche Vermehrung im Sauer toffgehalt des arteriellen Blutes bedingt selb t wenn verglichen mit dem Gesamthamoglobin ein Fall im Prozentgehalt des Sauerstoffs vorhanden ist BARCROFT nennt dann jedoch unter Hinweis auf die Steigerung des Hb auch bei unverandertem Druck wie z B bei Kohlenmonoxydvergiftung als den wahrscheinlich wesentlichen Punkt die durchschnittliche Sauerstoffkonzentration im Plasma der Kapillaren HURTADO MERINO und DELGADO (1945) heben den Zusammenhang zwischen dem Grad der Polyzythämie und dem Abfall der arteriellen O Hb Sattigung mit teigender Hohe hervor Sie weisen aber teils auch auf den etwas hoheren arteriellen

O_2-Gehalt der Höhenbewohner verglichen mit dem von Menschen bei Meereshöhe teils auf den geringeren Grad der Polyzythämie bei Lungenkranken in Meereshöhe mit gleich niedriger arterieller O-Hb-Sättigung wie bei Höhenbewohnern hin. Das wesentliche Problem des O-Mangels in Höhe *erscheint ihnen nicht auf die Menge des im Blute transportierten O* beschränkt. VERZÁR und Mitarbeiter (1915, 1917), die eingehend die ältere diesbezügliche Literatur diskutiert haben, halten vor allem auf Grund ihrer Untersuchungen in mittleren Höhen den arteriellen O-Gehalt die für die Erythropoiese wesentliche Grösse.

Eine vollständige Analyse dieses Problems verlangt sowohl die Kenntnis des Gesamtstatus der Erythropoiese wie auch die der O_2-Druck- und Konzentrationsverhältnisse in allen den Abschnitten und Systemen des Organismus, die unmittelbar mit der Erythropoiese im Zusammenhang stehen. Das ist praktisch unmöglich. Verhältnismässig einfach ist es jedoch, sich eine repräsentative Übersicht über hierbei wichtige hämatologische Daten zu verschaffen. Es wurden hier neben Hb-Konzentration und Hämatokrit vor allem auch das Gesamt-Hb bestimmt — die Resultante aller Hb-Bildungs- und Abbauprozesse. Damit wird der Begriff der Erythropoiese zwar stark eingeschränkt, jedoch auf einige Grössen, die funktionell gesehen und somit für Fragen der Regulation der Erythropoiese von grösstem Interesse sind. Mit Vorteil wählt man dabei Versuchsbedingungen, unter denen sich die Erythropoiese in einem relativen Gleichgewichtszustand befindet. In den vorliegenden Versuchen bei O_2-Drucken zwischen 760 und 50 mm Hg betrug die kürzeste Versuchszeit in 1000 m Höhe 24 Tage; in 6000 m Höhe hatte sich nach etwa 40 Tagen ein neues Hb-Niveau eingestellt. Da Ratten, die direkt 7000 m Höhe ausgesetzt wurden, nicht längere Zeit überlebten, befanden sich diese Tiere zunächst für mindestens 10 Tage in 6000 m Höhe. 8000 m Höhe überlebten nur 3 Tiere für 17 Tage. Die Versuchszeit in verschieden hohem O-Druck betrug durchschnittlich etwa 7 Wochen.

Ebenfalls über die O_2-Druck- und Konzentrationsverhältnisse im arteriellen und gemischten venösen Blut sind zuverlässige Aussagen möglich. Bei der Ratte sind allerdings derartige Messungen aus praktischen Gründen schwieriger und beschränken sich hier nur auf die Werte des arteriellen Blutes. Die Verhältnisse auf dem Gewebsniveau, wo das Zusammenspiel von O-Bedarf und O_2-Zufuhr ein äusserst komplexes Geschehen bildet, sind dagegen kaum zu überblicken. Alle bisherigen Versuche direkter O-Messungen dürften bestenfalls nur eine grobe Vorstellung der intrazellulären O-Druck- und Konzentrationsverhältnisse vermitteln. Auch alle entsprechenden Berechnungen beruhen auf einer Reihe von Annahmen, die im Einzelfall nur äusserst schwer zu überprüfen sind.

a) *Gesamthamoglobin bei Anderung des O_2 Drucks*

In Abb 1 i t als Ma s der Erythropoiese das Gesamt Hb/100 g Korper gewicht in Abhangigkeit vom O_2-Druck aufgetragen worden Der O_2-Druck der Einatmungsluft i t dabei innerhalb eines Druckbereichs von 760 bis etwa

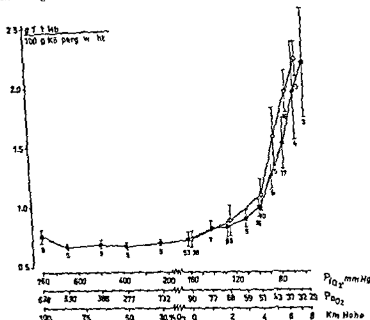


Abb 1

Gesamt Hamoglobin (als Hamoglobin/100 g Korpergewicht) von Ratten zweier Ge wichtsklassen nach vielwochentlichem Aufenthalt in verschiedenem O Druck. Mittel werte mit ± Standardabweichung und Anzahl der Tiere der jeweiligen Versuche Offene Symbole = Tiere bis zu 350 g, in 5000 m Hohe und daruber bis zu 300 g g s hl ssene Symbole = Tiere uber 351 g bzw 301 g Auf der Abszissenachse sind de trock ne) O Druck der Einatmungsluft, der arteri lle O_2-Druck sowie die entsprech ende O Konzen ration bzw die Hohe aufgetragen. Anderung des Skalenmasstabs bei hoherem O Druck als 160 mm Hg

50 mm Hg entsprechend reiner O_2-Atmung bei Normaldruck und *einem* in 8000 m Hohe herrschenden Hypoxiegrad verandert worden Der arterielle O_2-Druck wurde aus dem indirekt bestimmten alveolaren O Druck (s Arbeit XI) und der alveolaren arteriellen O Druckdifferenz berechnet Fur letztere wurde nach FARHI und RAHN (1955) bei Meere hohe und Hypoxie ein Wert von 6 mm Hg und fur *hohere* O_2-*Drucke* ein Wert von 35 mm Hg angenom men Die angegebenen *arteriellen* O_2-Drucke ent prechen Durch chnitt werten

die sich aus dem mittleren arteriellen O_2 Druckabfall herleiten Da sich das Gesamt Hb/100 g Korpergewicht vor allem bei starkerer Hypoxie, als gewichtsabhangig erwies, wurde das verfugbare Tiermaterial in 2 Gruppen verschiedener Gewichtsklassen aufgeteilt Bis zu 4000 m Hohe liegt die Gewichtsgrenze bei 350 g in 5000 m Hohe und daruber bei 300 g In 8000 m Hohe wurde das Gesamt Hb/100 g Korpergewicht aus den Gewichtswerten zu Versuchsbeginn berechnet, da hier das Gewicht während des Versuch teilweise stark abfiel Mit Ausnahme der Versuche in 8000 m Hohe befanden sich die Hb Werte der Tiere in einem relativen Gleichgewicht zustand

Aus dem Verhalten des Gesamt Hb/100 g Körpergewicht lässt sich folgendes erkennen Innerhalb eines weiten O_2 Druckbereichs von etwa 500 mm Hg ist die Erythropoiese praktisch unbeeinflusst Erst dann, wenn der O Druck der Einatmungsluft 150 mm Hg unterschreitet, steigt das Gesamt Hb und zwar bis zu etwa 100 mm Hg entsprechend 1000 m Hohe in relativ mässigem Umfang Statistisch zu sichernde Unterschiede des Gesamt Hb zwischen den beiden Gewichtsklassen finden sich nicht Mit weiter abfallendem O_2 Druck steigt das Gesamt Hb kraftig und in praktisch gleichem Umfang Die kleineren Tiere erreichen in 5000 m Hohe bei einem mittleren Gewicht von 282 g ein Gesamt Hb von 1,55 g/100 g Korpergewicht die grosseren Tiere bei einem mittleren Gewicht von 426 g ein Gesamt Hb von 1 23 g/100 g Korpergewicht In 6000 m Hohe werden Werte von 1 92 g Gesamt Hb/100 g Korpergewicht fur Tiere mit einem Durchschnittsgewicht von 264 g und 1 50 g Gesamt Hb/100 g Korpergewicht fur durchschnittlich 365 g schwere Tiere gefunden Diese Differenzen im Gesamt Hb/100 g Korpergewicht sind hochsignifikant Der Anstieg beruht im wesentlichen auf einer Anderung des Gesamt Hb und nicht auf einem Gewichtsabfall der mit etwa 10 % bei Überschreiten von 5000 m Hohe nur eine untergeordnete Rolle spielt Da die Sterblichkeit in 7000 m Hohe stark ansteigt, ist das zugangliche Material in 7000 und 8000 m sehr begrenzt Es ist aber dennoch ganz offenbar dass in 7000 m das Hb Bildungsvermogen nicht oder nicht wesentlich eingeschränkt ist Die Differenz zwischen den beiden Gewichtsklassen bleibt weiterhin bestehen 3 Tiere die 8000 m 17 Tage lang uberlebten steigerten weiterhin ihr Gesamt Hb Es ist bemerkenswert dass diese Tiere der hoheren Gewicht klasse zugehorten und dass bereits in 7000 m Hohe kein Tier der niedrigeren Gewichtsklasse im Gegensatz zu den schweren Tieren 90 Tage uberlebt hatte

b) Hamoglobinkonzentration bei Anderung des O Drucks

Abb 2 zeigt die Hb Konzentration in Abhängigkeit vom O Druck Die Anzahl der Werte ist hier geringer aber das prinzipielle Verhalten wird doch deutlich Auch hier hat hoher O Druck keinen oder einen ausserst geringen

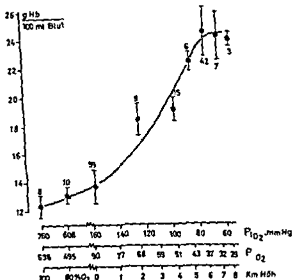


Abb. 2.

Hämoglobinkonzentration nach vielwöchentlichem Aufenthalt in verschiedenem O_2-Druck. Mittelwerte mit ± Standardabweichung und Anzahl der Tiere der jeweiligen Versuche Einteilung der Abszissenachse entsprechend Abb 1

Effekt. Im Gegensatz zum Gesamt Hb ist aber bereits in 2000 m Höhe die Hb Konzentration stark gestiegen und erreicht in 6000 m mit etwa 24 g% einen offensichtlichen Maximalwert

c) *Arterieller O_2-Gehalt bei Änderung des O_2 Drucks*

Aus den besprochenen Beziehungen zwischen O_2-Druck und Gesamt Hb bzw Hb Konzentration geht hervor dass sich die Erythropoiese im wesentlichen nur in einem solchen O_2-Druckbereich verändert, in dem auch gleichzeitig die arterielle O Hb Sättigung absinkt Es erhebt sich hier die nachfolgend behandelte Frage wie sich dabei der arterielle O_2-Gehalt verhält

Der arterielle O_2-Gehalt ergibt sich aus der Hb Konzentration dem O_2-Bindungsvermögen des Hb der O Hb Sättigung und der physikalisch gelösten O_2-Menge In Abb 3 ist der arterielle O_2-Gehalt bei verschiedenem O_2-Druck dargestellt Die diesen Berechnungen zugrunde liegenden Einzeldaten sind Tab 1 zu entnehmen Die arterielle O Hb-Sättigung ist mit Ausnahme der Versuche in 80% und 100% O_2 und 8000 m Höhe, für die eine 100%ige bzw. 25%ige O Hb-Sättigung eingesetzt wurde direkt bestimmt worden Die Hb-Konzentration des Schwanzblutes liegt durchschnittlich 13% über der des

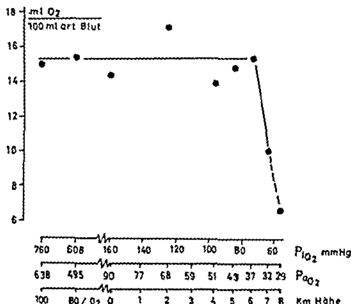


Abb 3

Arterieller O Gehalt von Ratten nach vielwochentlichem Aufenthalt in verschiedenem O_2 Druck berechnet aus Hamoglobinkonzentration, O Hb Sattigung und physikalisch gelostem O Fur die O Hb Sattigung in 8000 m Hohe wurde ein (extrapolierter) Wert von 25 % eingesetzt Einteilung der Abszissenachse entsprechend Abb 1

Tab I

Mittelwerte des O_2 Drucks der O Hb Sattigung und der Hamoglobinkon entration des arteriellen Blutes und der daraus berechnete chemisch gebundene physikalisch geloste und gesamte O_2 Gehalt bei Normaltieren und Ratten nach vielwochentlichem Aufent halt in hohem und niedrigem O_2 Druck Die zugrundeliegenden Werte wurden den Arbeiten VII IX und XI entnommen Bei dem arteriellen O_2 Druck wurde vom mitt leren Verlauf ausgegangen Die Hamoglobinkon entration bei Meereshohe wurde aus den Ausgangswerten aller Tiere vor Hypoxie bzw Hyperoxie berechnet

	Pa O mm Hg	Sa O %	Hb Konz g %	O_2 ml/100 ml art. Blut		
				chem geb	phys gel	total
100 % O	638	100	10 7	14 02	1 91	15 93
80 % O_2	495	100	11 4	14 93	1 48	16 41
0 m	90	91	11 9	14 18	0 27	14 45
2000 m	68	83	16 0	17 00	0 20	17,20
4000 m	51	65	16 6	13 81	0 15	13,°6
5000 m	43	59	19 4	14 65	0 13	14 78
6000 m	37	56	21,2	15 20	0 11	15 31
7000 m	32	39	21 0	10 48	0 10	10 ‿8
8000 m	29	25	20 8	6 66	0 09	6 75

arteriellen Blutes (s Arbeit I) Da die Hb Konzentrationen vom Schwanzblut bestimmt worden ind wurde eine ent prechende Korrektur vorgenommen Fur das O Hb Bindungsvermogen von Normaltieren war ein mittlerer Wert von 1,31 ml/g Hb und fur das von Hypoxietieren von 1,28 ml/g Hb ge funden worden Die physikalisch geloste O Menge wurde mit 0 003 ml/ml Blut und 100 mm Hg angenommen

Aus Abb 3 ist klar zu erkennen dass bis zu einem O Druck entsprechend 6000 m Hohe der arterielle O_2-Gehalt auf einem konstanten Niveau gehalten wird Er sinkt in 7000 m Hohe auf 2/3 des Normalwertes ab und betragt in 8000 m Hohe nur etwa 1/3 des Normalwertes

d) *Be iehung wischen arteri ller O_2Hb Sattigung und Gesamthämoglobin*

Die Veranderungen der Erythropoiese fuhren nach dem Besprochenen dazu dass der arterielle O Gehalt bis zu einem Hypoxiegrad entsprechend 6000 m Hohe konstant gehalten wird Liegt das Regulationsziel der Erythropoiese unter Hypoxie lediglich darin einen normalen arteriellen O Gehalt beizu behalten so sollte der Anstieg des Gesamt Hb dem Abfall der O Hb Sattigung entsprechen Sinkt beispielsweise die O Hb Sattigung um 50% so muss das Gesamt Hb um 100% teigen u s w In wie weit der gefundene Anstieg des Gesamt Hb mit den Berechnungen ubereinstimmt soll nachfolgend behandelt werden

Wie bereits oben gezeigt (Abb 1) teigt das auf das Korpergewicht be zogene Ge amt Hb (g Hb/100 g Korpergewicht) unter Hypoxie von kleinen Tieren starker als das grosserer Tiere Eine Aufteilung der Werte nach Ge wicht kla en i t deshalb notwendig Der begrenzte Umfang des Materials macht jedoch eine d rartige Aufteilung in eine gro sere Zahl von Einzelklas en un moglich Die allgemeine Sachlage unter den ver chiedenen Versuchs bedingungen lasst sich aber dennoch zeigen wenn man vom mittleren Ver halten des Gesamt Hb in Abhangigkeit vom Korpergewicht das durch lineare Regression linien beschrieben werden kann ausgeht und dieses miteinander vergleicht Die Gleichungen derartiger Regressionslinien sind in Tab II zu ammengestellt In Abb 4 sind die auf die e Wei e von einer grösseren Anzahl von Normaltieren (Arbeit II) berechneten Mittelwerte gleich 100 ge setzt und die prozentuale Abweichung davon unter Hypoxie von 275 g 350 g und 450 g schweren Tieren dargestellt War die Zahl der Einzelwerte wie in 1000 m 3000 m und 8000 m Hohe zu gering um Regression linien zu be rechnen wurden die Mittelwerte der entsprechenden Tiere der am nachsten liegenden Gewicht gruppe zugeordnet. In dieser Abbildung ist ferner aus gehend von der sinkenden arteriellen O Hb Sättigung unter Hypoxie der

Tab II

Korrelation zwischen Gesamt Hb (y) und Korpergewicht (x) bei Meereshohe und verschiedenen Hypoxiegraden Gleichungen der Regressionslinien mit ± Standardabweichung Korrelationskoeffizienten und Signifikanz der Korrelationskoeffizienten. Den Berechnungen in Meereshohe liegen die Werte von 378 140—500 g schweren Kontrolltieren zugrunde (Arbeit II) n = Anzahl der Werte

Hohe m	n	$y = a + bx \pm \sigma$	$r \pm \varepsilon r$	p
0	378	$y = 0\,240 + 0\,00657\,x \pm 0\,175$	$0\,96 \pm 0\,004$	$< 0\,001$
2000	16	$y = 1\,979 + 0\,00338\,x \pm 0.350$	$0\,45 \pm 0\,205$	0 05—0 01
4000	26	$y = 1\,399 + 0\,00632\,x \pm 0\,318$	$0\,76 \pm 0\,083$	$< 0\,001$
5000	11	$y = 2\,528 + 0\,00636\,x \pm 0\,5?8$	$0\,72 \pm 0\,150$	0 01—0 001
6000	33	$y = 2\,706 + 0\,00818\,x \pm 0\,740$	$0\,56 \pm 0\,119$	$< 0\,001$
7000	7	$y = 6\,233 - 0\,00140\,x \pm 0\,813$	$-0\,07 \pm 0\,406$	$> 0\,05$

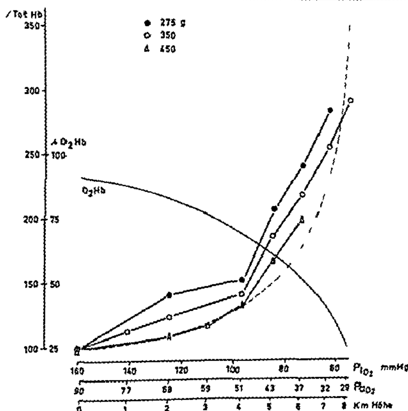


Abb 4

Anstieg des Gesamt Hamoglobin in % des Ausgangswertes (= 100) von 275 g 350 g und 450 g schweren Ratten in Hypoxie Der gestrichelte Kurvenverlauf gibt die Menge Hamoglobin an die notwendig ware um das durch den Abfall der O Hb Sattigung bedingte Absinken des arteriellen O_2 Gehalts zu kompensieren. Die Kurve der O Hb-Sattigung entspricht einem mittleren Verlauf Einteilung der Abszissenachse entsprechend Abb 1

berechnete Kurvenverlauf des Gesamt Hb um den O Hb Sattigungsabfall durch eine gesteigerte Hb Konzentration zu kompensieren wiedergegeben

Aus der Betrachtung des annahrungsweise hyperbolischen Kurvenverlaufs der berechneten Hb Menge geht hervor dass bis zu etwa 6000 m Hohe die Hb Menge etwa verdoppelt werden mu s um den Abfall der O Hb Sattigung auszugleichen Daruber hinaus wird mit jedem kleineren Druckabfall die zu bildende Hb Menge immer gros er und unabhängig davon dass die Hb Kon zentration nicht beliebig ansteigen kann und andere Faktoren determinierend sein mögen lasst sich auch ein endlicher Grenzwert der Hb Bildung absehen

Die gefundenen Werte des Gesamt Hb weichen von dem berechneten Kur venverlauf teilweise nicht unerheblich und systematisch ab Je kleiner die Tiere sind desto grosser ist die Abweichung Lediglich die 450 g schweren Tiere folgen bis zu 4000 m Hohe den Berechnungen zufriedenstellend Die 350 g und 275 g schweren Tiere zeigen bereits bis zu 4000 m um 10—20% hohere Hb Werte In 5000 m weichen die 275 g Tiere mit uber 50% und in 6000 m mit uber 60% stark vom berechneten Wert ab Auch die 450 g Tiere haben in 5000 m deutlich hohere Werte die 350 g Tiere nehmen eine Mittel stellung ein In 6000 m naheren sich die grossten Tiere dem berechneten Wert nicht jedoch die mittleren Tiere und kleinsten Tiere Die 8000 m Werte von 3 nicht volladaptierten Ratten deuten an welche hohe Hb Bildungslei tung diese Tiere bei einer extrem niedrigen O Hb Sattigung (25% — extrapolierter Wert) noch vollbringen

Die Entscheidung daruber ob die gefundenen Werte in ihrem Verlauf am ehesten zusammengesetzten linearen oder gekrummten Kurven entsprechen muss offen bleiben Dazu sind umfangreichere Untersuchungen notwendig Eine wesentliche Rolle dabei spielen u a die Werte in 4000 m Um diese zu sichern sind zu verschiedenen Zeitpunkten (1958 1959 1960) zu ammen 1 Tiergruppen in dieser Hohe untersucht worden Die Resultate waren trotz dieser ausgedehnten Versuchsdauer und der damit verbundenen stärkeren Inhomogenitat des Tiermaterials gut reproduzierbar

Dass es tatsachlich sinnvoll sein kann die Frage nach der Art der Kurven verlaufe zu di kutieren wird deutlich wenn man die nachfolgend behandelten Veranderungen des Blutvolumens in Abhangigkeit vom Grad der Hypoxie betrachtet

e) Blutvolumen bei Anderung des O_2 Drucks

Das vorhandene Material ist hier relativ so gering dass eine ins einzelne gehende Darstellung nicht moglich i t und in Abb 5 nur das grund atzliche Verhalten des Blutvolumens in Abhangigkeit vom O_2-Druck der Einatmungs luft gezeigt werd n kann Wie in Abb 4 wurden die Normalwerte bei Meeres

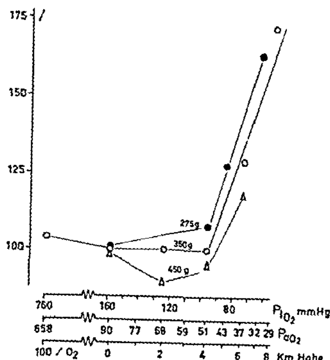


Abb 5

Abweichen des Blutvolumens unterschiedlich schwerer Ratten in % vom Werte bei Meereshohe (= 100) bei verschiedenem O Druck Einteilung der Abszissenachse entsprechend Abb 1

Tab III

Korrelation zwischen Blutvolumen (y) und Korpergewicht (x) bei Meereshohe und verschiedenen Hypoxiegraden Gleichungen der Regressionslinien mit ± Standardabweichung Korrelationskoeffizienten und Signifikanz der Korrelationskoeffizienten Bei den Normalwerten wurden Tiere unter 200 g Korpergewicht die eine etwas geringere Hb Konzentration als altere Tiere haben konnen (Arbeit II) sowie solche mit niedrigerer Hb Konzentration als 11 g % ausgeschlossen n = Anzahl der Werte

Hohe m	n	$y = a + bx \pm \sigma$	$r \pm \varepsilon r$	p
0	218	y = 3 51 + 0 0581 x ± 2 28	0 87 ± 0 016	< 0 001
2000	9	y = 14 37 + 0 0?71 x ± 1 13	0 50 ± 0 264	0 10—0 05
4000	15	y = 9 89 + 0 0404 x ± 1 73	0 81 ± 0 091	< 0 001
6000	29	y = 15 34 + 0 0435 x ± 2 60	0 72 ± 0 090	< 0 001
7000	7	y = 25 07 + 0 0247 x ± 2 55	0 27 ± 0 378	> 0 05

hohe gleich 100 gesetzt und die Abweichung davon in Prozent dargestellt Dabei wurden analog Abb 1 die Regressionslinien vom Blutvolumen in Abhangigkeit vom Korpergewicht berechnet Diese sind Tab III zu entnehmen

In Abb 5 sind so erhaltene Werte fur 275, 350 und 450 g schwere Tiere dargestellt In 2000 m Hohe waren nur Tiere von mehr als 350 g Gewicht vorhanden umgekehrt in 7000 m nur kleinere als 350 g schwere Tiere In 5000 m lag eine Gruppe von Ratten den 275 g schweren Tieren und in 8000 m den 350 g schweren Tieren am nachsten

Das Verhalten des Blutvolumens zeigt ein charakteristisches Bild In hohem O Druck und in Hypoxie bis zu einer Hohe entsprechend 4000 m sind keine wesentlichen Veranderungen nachzuweisen in Hypoxie besteht lediglich teilweise eine gewisse Tendenz zu einem Abfall Mit weiter sinkendem O-Druck steigt dann das Blutvolumen angenahert linear wobei sich unterschiedlich grosse Tiere deutlich voneinander unterscheiden

Das vorliegende Material lasst aber noch weitere Einzelheiten erkennen die auf die biologische Bedeutung der Blutvolumensregulation unter Hypoxie hinweisen Das wird aus dem Vergleich der Korrelationskoeffizienten deutlich wenn man vom selben Material gleichzeitig die Regressionslinien für das Gesamt Hb und das Blutvolumen in Abhangigkeit vom Korpergewicht berechnet In Meereshohe ist das Blutvolumen etwas schlechter als das Gesamt Hb zum Korpergewicht (Korrelationskoeffizient r = 0 87 gegenuber 0 90) korreliert. Das beruht auf den Schwankungen der Hb Konzentration (biologische Variationen und Methodenfehler) und damit verbunden des Blutvolumens Anders in Hypoxie Die Korrelationskoeffizienten des Blutvolumens sind etwa denen des Gesamt Hb gleich in 2000 m 0 50 gegenuber 0 49 in 4000 m 0 81 gegenuber 0 83 in 6000 m 0 72 gegenuber 0 60 In 7000 m findet sich kein Zusammenhang zum Korpergewicht

f) Der Einfluss des Korpergewichts auf Gesamthamoglobin und Blutvolumen

Im Vorangegangenen ist die Einwirkung des O Drucks also eines exogenen Faktors auf verschiedene Grossen der Erythropoiese behandelt worden Dabei zeigte es sich dass die Reaktionsweise der Erythropoiese auch von einem endogenen Faktor dem Alter bzw der Tiergrosse abhangt Es mag naheliegend erscheinen die relativ starkere hypoxische Reaktion kleinerer Tiere auf den relativ hoheren Stoffwechsel dieser Tiere zuruckzufuhren Da der Stoffwechsel dieser Tiere nicht gemessen worden ist kann sich hier eine solche Auffassung nur auf indirekte Daten stutzen Eine derartige Möglichkeit besteht darin die gemessenen Grossen mit anderen Korperparametern zu vergleichen Folgen die Veranderungen des Gesamt Hb bzw des Blutvolumens am ehesten dem Korpergewicht, spricht das gegen einen unmittelbaren Einfluss des Stoffwechsels Folgen aber Gesamt Hb und Blutvolumen am ehesten der Korperoberflache ist ein Einfluss des Stoffwechsels naheliegend da

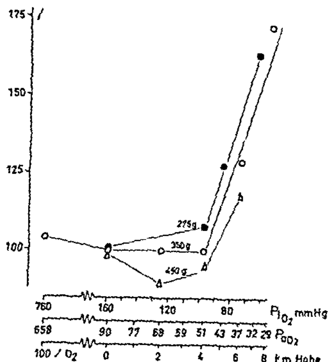


Abb. 5

Abweichen des Blutvolumens unterschiedlich schwerer Ratten in % vom Werte bei Meereshöhe (= 100) bei verschiedenem O_2-Druck. Einteilung der Abszissenachse entsprechend Abb. 1

Tab. III

Korrelation zwischen Blutvolumen (y) und Körpergewicht (x) bei Meereshöhe und verschiedenen Hypoxiegraden. Gleichungen der Regressionslinien mit ± Standardabweichung, Korrelationskoeffizienten und Signifikanz der Korrelationskoeffizienten. Bei den Normalwerten wurden Tiere unter 200 g Körpergewicht, die eine etwas geringere Hb-Konzentration als ältere Tiere haben können (Arbeit II), sowie solche mit niedrigerer Hb-Konzentration als 11 g % ausgeschlossen. n = Anzahl der Werte

Höhe m	n	$y = a + bx \pm \sigma$	$r \pm \varepsilon r$	p
0	218	$y = 3.51 + 0.0581\,x \pm 2.28$	0.87 ± 0.016	< 0.001
2000	9	$y = 14.37 + 0.0271\,x \pm 1.13$	0.50 ± 0.264	0.10—0.05
4000	15	$y = 9.89 + 0.0404\,x \pm 1.73$	0.81 ± 0.091	< 0.001
6000	29	$y = 15.34 + 0.0435\,x \pm 2.69$	0.72 ± 0.090	< 0.001
7000	7	$y = 25.07 + 0.0747\,x \pm 2.55$	0.27 ± 0.378	> 0.05

höhe gleich 100 gesetzt und die Abweichung davon in Prozent dargestellt. Dabei wurden analog Abb. 4 die Regressionslinien vom Blutvolumen in Abhängigkeit vom Körpergewicht berechnet. Diese sind Tab. III zu entnehmen.

Tab V

Gesamt Hamoglobin und Blutvolumen von 275 g 350 g und 450 g schweren Ratten in Meereshohe und verschiedenen Hypoxiegraden bezogen auf Korpergewicht und Kor peroberflache Die Korperoberflache wurde nach LEE (1929) berechnet.

Hohe m	Korper gew g	Tot. Hb g	g Tot. Hb / 100 g Kor gew	g Tot. Hb / 100 cm Kor ober flache	Blutvol ml	ml Blutvol. / 100 g Kor gew	ml. Blutvol. / 100 cm Kor ober flache
0	275	2.06	0.75	0.56	19.5	7.1	5.4
	350	2.55	0.73	0.60	23.9	6.8	5.7
	450	3.21	0.71	0.65	29.7	6.6	6.0
2000	275	2.91	1.06	0.80	—	—	—
	350	3.16	0.90	0.75	23.8	6.8	5.6
	450	3.50	0.78	0.71	26.6	5.9	5.4
4000	275	3.14	1.14	0.85	21.0	7.6	5.8
	350	3.61	1.03	0.86	24.0	6.8	5.7
	450	4.24	0.94	0.86	28.1	6.2	5.7
5000	275	4.28	1.56	1.18	—	—	—
	350	4.75	1.36	1.12	—	—	—
	450	5.39	1.20	1.10	—	—	—
6000	275	4.96	1.80	1.35	27.3	9.9	7.5
	350	5.57	1.59	1.32	30.6	8.7	7.2
	450	6.39	1.42	1.30	34.9	7.8	7.1
7000	250	5.88	2.35	1.72	31.2	12.5	9.1
	300	5.81	1.94	1.51	32.5	10.8	8.5

Allerdings sind in 6000 m Hohe gewisse Abweichungen festzustellen die in 7000 m noch deutlicher hervortreten

g) *Zur Entwicklung der Polyzythamie bei Hypoxie*

Der Entwicklung der Hohenpolyzythamie wie sie am Verlauf der taglich gebildeten Hb-Menge zu verfolgen ist, kommt aus zwei Grunden besonderes Interesse zu Fur sich betrachtet gibt sie einen Einblick in die Dynamik der Hb-Bildung und das Leistungsvermogen des hämatopoietischen Gewebes zu sammen mit dem Hb-Bildungsverlauf bei Anamie ermoglicht sie eine ver gleichende qualitative und quantitative Beurteilung dieser beiden Stimulations formen der Erythropoiese

Je nach Hypoxiegrad und in Abhangigkeit davon ob die Erythropoiese zuvor durch geringere Hypoxie oder Anämie auf ein hoheres Aktivitats-

auch der Stoffwechsel am besten zur Korperoberflache, jedenfalls bei einem Material mit sehr verschiedener Tiergrosse korreliert ist

Mit Vorteil kann fur solche Untersuchungen die sogenannte allometrische Darstellungsweise verwendet werden Dabei wird der Zuwachs *einer Grösse* Y mit der Anderung einer Grosse X durch die Gleichung $Y = a \; X^b$, oder anders ausgedruckt $\log Y = \log a + b \; \log X$ beschrieben Das bedeutet, dass Y und X bei logarithmischer Darstellungsweise auf einer Geraden liegen vorausgesetzt dass sie in einem konstanten Verhaltnis zueinander stehen Die Konstante b gibt dabei an in welchem Verhaltnis sich Y in Abhangigkeit von X andert b = 1 2/3 u s w bedeutet also eine Veranderung im Verhaltnis 1 1, 2 3 u s w

Tab IV

Beziehung zwischen Gesamt Hamoglobin bzw Blutvolumen (y) und Korpergewicht (x) entsprechend $y = a \; x^b$ bei Meereshohe und verschiedenen Hypoxiegraden n = Anzahl der Bestimmungen Die 8 Werte fur das Gesamt Hamoglobin in Meereshohe reprasentieren 378 140—500 g schwere Tiere (Arbeit II) die nach ihrem Gewicht in 8 Gruppen aufgeteilt worden waren

Hohe m	n	Gesamt Hamoglobin	n	Blutvolumen
0	8	$y = - 6\ 94 \; x^{0\ 88}$	218	$y = - 5\ 40 \; x^{0\ 83}$
2000	16	$y = - 3\ 67 \; x^{0\ 42}$	9	$y = + 1\ 80 \; x^{0\ 44}$
4000	26	$y = -11\ 38 \; x^{0\ 63}$	15	$y = - 1\ 34 \; x^{0\ 59}$
5000	11	$y = - 2\ 88 \; x^{0\ 45}$	—	—
6000	33	$y = - 2\ 72 \; x^{0\ 46}$	29	$y = + 1\ 66 \; x^{0\ 50}$
7000	7	$y = + 7\ 63 \; x^{-0\ 05}$	7	$y = + 12\ 48 \; x^{0\ 17}$

In Tab IV sind die allometrischen Gleichungen fur die Korrelationen von *Gesamt Hb und Blutvolumen zum Korpergewicht fur Tiere in Meeres*hohe und verschiedenen Hypoxiegraden zusammengestellt Aus dem Vergleich *der Exponenten* b *in Meereshohe und Hypoxie lassen sich wesentliche Diffe*renzen erkennen b fallt von 0 88 bzw 0 83 in Meereshohe auf Werte zwischen 0 42—0 63 in 2000—6000 m und in 7000 m auf etwa 0

Die Bedeutung dieser Anderung wird deutlicher wenn man das Gesamt Hb bzw Blutvolumen verschieden grosser Tiere auf Korpergewicht und Körperoberflache bezieht und die gefundenen Relationswerte miteinander vergleicht Tab V zeigt einen derartigen Vergleich Die Korperoberfl iche wurde nach der von Lee (1929) gefundenen Gleichung fur die Korperoberflache in Abhangigkeit vom Gewicht Korperoberflache = 12 51 Korpergewicht$^{0\ 60}$ berechnet In Meereshohe mogen Gesamt Hb und Blutvolumen eher dem Korpergewicht als der Korperoberflache folgen, die Differenz ist aber nicht gross In Hypoxie ist dagegen die *Körperoberflache klar der bessere Parameter*

3 Cobalt

Eine der wenigen Möglichkeiten, die Erythropoiese zu stimulieren, ohne gleichzeitig den arteriellen O_2-Druck oder O_2-Gehalt zu vermindern, bietet die Zufuhr von Cobalt (Literatur s. Grant und Root 1952). Nach den Untersuchungen von Goldwasser et al. (1957) sowie Jacobson und Goldwasser (1958) bildet Cobalt erythropoietisch aktive Substanzen oder setzt diese frei, die ihrerseits den eigentlichen Stimulus der Erythropoiese darstellen. Änderungen im Auftreten dieser Substanzen unter verschiedenen Bedingungen, u. a. nach Nephrektomie, sind von Jacobson und Mitarbeitern zur Lokalisation der Organe herangezogen worden, die diese Substanzen bilden oder freisetzen. White et al. (1960) haben weiterhin den erythropoiese-stimulierenden Effekt von 5 μM $CoCl_2$ gemessen an der Höhe der Fe^{59}-Inkorporation bei der fastenden Ratte als biologischen Standard für andere erythropoietisch aktive Substanzen verwendet.

Der erythropoiese-stimulierende Effekt des Plasma von Ratten nach Injektion von 25 μM $CoCl_2$/100 g Körpergewicht übertrifft den im Plasma nach einer massiven Blutung zu findenden um das Vielfache (Jacobson und Goldwasser 1958). Das ist insofern bemerkenswert, als die Erythrozytenwerte der Ratte nach vielfach wiederholter Injektion von Cobalt nur etwa verdoppelt werden (Berlin 1951). Hämolytische Reaktionen oder eine verkürzte Lebenszeit unter Cobalt-Gabe gebildeter Erythrozyten können jedoch zu einem wesentlich höheren Erythrozytenumsatz führen ohne entsprechenden Anstieg der Gesamterythrozytenmenge. Nach den Untersuchungen von Van Dyke et al. (1955) ist die Lebenslänge unter Cobalt-Gabe gebildeter Erythrozyten jedoch normal; über hämolytische Reaktionen scheint nichts bekannt zu sein.

In den eigenen Untersuchungen (Arbeit XII) wurde die Entwicklung der Cobalt-Polyzythämie bei einer Dosierung von 0,4, 0,6 und 0,8 mg $CoCl_2$/100 g Körpergewicht und Tag verfolgt. Aus dem Verhalten der spezifischen bzw. gesamten Aktivität zuvor *in vivo* radioaktiv gezeichneten Hämins wurde außerdem versucht, Aufschluss über eventuelle hämolytische Reaktionen zu erhalten. 3wöchentliche Zufuhr von 0,4 *mg $CoCl_2$/100* g Körpergewicht führte bei unverändertem Gesamt-Hb zu einem signifikanten Anstieg der Hb-Konzentration und Retikulozyten. Hämolytische Reaktionen waren nicht nachzuweisen. Während der nachfolgenden 3wöchentlichen Periode mit einer täglichen Zufuhr von 0,6 mg $CoCl_2$/100 g Körpergewicht entwickelte sich eine echte Polyzythämie mit maximalen Retikulozytenwerten und einem signifikant erhöhtem Gesamt-Hb. Nach weiter 6wöchentlicher Cobalt-Behandlung mit 0,8 mg $CoCl_2$/100 g Körpergewicht wurden bei kontinuierlich steigendem Gesamt-Hb absolute und relative Blutwerte, wie sie bei Tieren

niveau gebracht worden ist, werden unterschiedliche Bildungsverlaufe gefunden, die in Arbeit VII beschrieben sind und auf die verwiesen ei Es sollen hier nur einige qualitative und quantitative Unterschiede zu dem Bildungsverlauf bei Anamie hervorgehoben werden Auch bei einem geringeren hypoxischen Reiz entsprechend 3000 m und 1000 m Hohe erreicht die Hb Bildung auf ihrer Hohe das 3—4 fache der Norm bei akuter Anämie wird ein etwa verdoppelter Wert gefunden In Anämie setzt die Hb Bildung etwa am 3 Tag nach der Blutentnahme ein, in Hypoxie wird bereits nach 2 Tagen ein scharf ausgebildetes Maximum gefunden mit Ausnahme der Versuche, bei denen bereits eine erhohte Erythropoiese vorlag

Diese erste Phase der Hypoxie bei der die Hb Konzentration des Blutes kraftig ansteigt, ist in Arbeit VIII naher studiert worden Aus dem Verhalten der spezifischen Aktivitat zuvor *in vivo* radioaktiv gezeichneten Hämins *und dem Gesamt Hb* kann geschlossen werden dass wahrend der ersten beiden Tage in Hypoxie trotz dieser kraftigen Hb Synthese kein Hb aus dem Gewebe in die Zirkulation ubertritt Eine hamolytische Reaktion die VERZAR (1917) als moglichen Bildungsreiz der Erythropoiese diskutiert hat, war nicht zu erkennen

h) *Zur Ruckbildung der Polyzythamie nach Hohenaufenthalt*

Die Readaptation eines hohenpolyzythamischen Organismus an Meereshohe ist mit einer Reihe hamatologischer Fragen beispielsweise den eines vermehrten Zellabbaus oder der Hemmung der Zellproliferation verknupft Die Readaptation lasst sich aber auch im Hinblick auf die O_2 Versorgung des Gewebes betrachten Dabei ergibt sich die interessante Situation dass die durch Hypoxie hervorgerufene gesteigerte Kapillarisation des Gewebes nach Übergang auf Meereshohe eine bessere O Versorgung des Gewebes als normal *moglich erscheinen lasst Hangen* die regulatorischen Prozesse der Erythro*poiese von der* adaquaten O Versorgung des Gewebes ab so sind hier Voraussetzungen dafur gegeben dass die O Kapazitat des Blutes kompensatorisch unter den Normalwert absinkt

In Arbeit X wurde der Abfall der absoluten und relativen Blutwerte von hohenpolyzythamischen Ratten nach Übergang auf Meereshohe untersucht Eine vorubergehende relative Anamie etwa 10 Tage nach Ende des Hohenaufenthalts kann zwar in dem genannten Sinne gedeutet werden Solange jedoch die kapillare Durchblutung bzw der O Druck des Gewebes selbst nicht bekannt sind ist es nicht moglich daraus mit Sicherheit einen funktionellen Zusammenhang zwischen der Hohe der O Kapazitat des Blutes und dem Grad der Kapillarisation des Gewebes herzuleiten

daran festgehalten dass die Steigerung der Erythropoiese unter Hypoxie direkt durch den arteriellen O Druck ausgelö t wird BARCROFT begründet dis mit dem beim Hohenbewohner hoheren arteriellen O-Gehalt als normal HURTADO et al (1945) haben diesen Befund bestätigen können Ein ahnliches Verhalten haben auch OTIS und HUSSON (1956) beim zyanotischen Herzkranken in Meereshohe bis zu einer reduzierten O Hb Sättigung von etwa 70 % gefunden

In den eigenen Untersuchungen erscheint der arterielle O_2-Gehalt in 2000 m Hohe ebenfalls ungewohnlich hoch im ubrigen sind aber keine sicheren Au sagen zu diesem Punkt moglich Verglichen mit den von Geburt aus in Hohe lebenden bzw herzkranken Menschen ist hier die Expositionszeit namlich relativ o kurz dass ein unmittelbarer Vergleich ausgeschlossen ist und die Moglichkeit offen bleiben muss ob bei langerer Versuchszeit nicht doch hohere Werte zu finden gewesen waren In einem etwa 4 wochentlichen Versuch am Menschen bis zu 6000 m Hohe fanden HOUSTON und RILEY (1947) ebenfalls keinen erhohten arteriellen O-Gehalt Nach REYNAFARJE LOZANO und VALDIVIESO (1959) hat der Mensch nach 1 jahrigem Aufenthalt in 4500 m Höhe noch nicht eine dem standigen Hohenbewohner entsprechende maximale Polyzythamie entwickelt

2 Aus dem offenbar fehlenden direkten Zusammenhang zwischen arteriellem O-Druck und Erythropoiese ergibt sich noch nicht zwingend dass der O auf die Erythropoiese uber Prozes e die sich im Gewebe abspielen ein wirkt Es ist namlich a) denkbar dass im anoxischen Blut selbst Verbindungen gebildet werden die die Erythropoiese stimulieren HIRSJARVI (1953) hat diese Vorstellung fur die jedoch endgultige Beweise noch ausstehen, eingehend diskutiert Es mag aber b) auch die Moglichkeit bestehen da s im Bereich des venosen Gefassystems analog den Chemorezeptoren des arteriellen Gefassystems (Carotis Aorta) O Druck empfindliche Rezeptoren vorhanden sind die spezifische Wirkungen auf die Erythropoiese ausuben Anatomische Befunde lassen prinzipiell auch die Existenz derartiger *venoser* Chemorezeptoren moglich erscheinen (Glomus pulmonale KRAHL 1962) Auch der kraftige Anstieg des Herzminutenvolumens bei selektiv herabgesetztem O Druck des Blutes der a pulmonalis (HILPERT BARBEY und BARTELS 1962) mag funktionell gesehen die Vor *tellung* venoser Chemorezeptoren stutzen Bislang liegen jedoch keinerlei Befunde vor die auf eine Bedeutung solcher jedenfalls zentraler Chemorezeptoren für die Erythropoiese hinweisen konnten

3 Im nachfolgenden Abschnitt soll erortert werden ob und was dafur spricht da s die Erythropoiese unmittelbar mit einer adaquaten O Ver or gung des Gewebes zu ammenhangt Das ware einfach, wenn bekannt wäre

in etwa 1000 m Höhe gefunden werden, erreicht. Trotz dieser unverändert *hohen Hb-Bildungsleistung fiel die Retikulozytenkonzentration auf Normalwerte ab.*

Diskussion zu II

Ziel der vorliegenden Arbeit ist, Aufschluss über Grössen und Prozesse zu erhalten, die die Bildungsleistung der Erythropoiese bestimmen. Besonderes Interesse kommt dabei der Funktion des Hb als Beförderungsmittel des O zum Gewebe zu, da angenommen werden kann, dass die Grösse der Erythropoiese davon *abhängt, in wie weit die O_2-Zufuhr zum Gewebe dessen O*-Bedarf entspricht. Dieser relativ einfachen Grundkonzeption von der Regulation der Erythropoiese stehen jedoch eine Reihe widerspruchsvoller Auffassungen über die Wirkung verschiedener Reize auf die Blutbildung entgegen, die sich allerdings im allgemeinen nicht auf quantitative Messungen stützen können. Einige überwiegend quantitative Studien der Erythropoiese unter verschiedenen Bedingungen, die derartige Fragen klären mögen, liegen hier vor. Das differenzierteste Bild zeigen dabei die Untersuchungsresultate bei verschiedenem O_2-Druck der Einatmungsluft, mit deren Besprechung begonnen werden soll.

1. Bei den Untersuchungen der Erythropoiese in Abhängigkeit vom O-Druck der Einatmungsluft ist es zweckmässig, zunächst festzustellen, ob ein direkter Zusammenhang zwischen der Erythropoiese und dem *arteriellen O_2*-Druck besteht. Ist dieses der Fall, liegen Problemstellungen vor, deren Bearbeitung nach ähnlichen Gesichtspunkten erfolgen kann wie beispielsweise die Behandlung der vom arteriellen O_2-Druck abhängigen Atemreaktionen.

Die *in einem weiten* Bereich hohen O_2-Drucks unveränderte Hb-Menge und die strenge Zuordnung der Veränderungen der Hb-Menge zur absin*kenden O_2Hb-Sättigung unter Hypoxie sprechen nicht für eine* direkte Einwirkung des arteriellen O_2-Drucks auf die Erythropoiese. Die Vorstellung eines solchen Zusammenhangs wird man gänzlich fallen lassen müssen, wenn man die gesteigerte Erythropoiese unter Bedingungen in Betracht zieht, bei denen der arterielle O_2-Druck unverändert ist, der arterielle O_2-Gehalt jedoch herabgesetzt ist (Anämie, CO-Vergiftung, Methämoglobinämie), oder wenn man umgekehrt die Hemmung der Erythropoiese bei höherem O-Gehalt als normal (Readaptation des höhenadaptierten *Organismus an Meereshöhe*) berücksichtigt.

Obwohl Barcroft (1927) die letztgenannten Befunde auch als Argument gegen die Vorstellung unmittelbar durch den arteriellen O-Druck hervor*gerufener Veränderungen der Erythropoiese* genannt hat, hat er dennoch

jedoch fur eine solche Vorstellung sprechen Diese Korrelation zur Korperoberflache in Hypoxie unterscheidet sich bemerkenswert von den Verhältnissen bei Meereshohe Nach den vorliegenden Daten folgen Gesamt Hb und Blutvolumen von Kontrolltieren eher dem Korpergewicht (Anstieg des Gesamt Hb bzw Blutvolumens im Verhaltnis zum Anstieg des Körpergewichts = 0 88 bzw 0 83) Bei einem grosseren Material mit verschiedenen Tierspezies wird dieser enge Zusammenhang zum Körpergewicht noch klarer BRODY (1945) fand fur den Anstieg des Blutvolumens in Abhängigkeit vom Körpergewicht einen Zusammenhang entsprechend Blutvolumen = 0 0507 Körpergewicht 0 99 (Werte von Ratte Huhn Hund, Stier) ADOLPH (1949) fur das Gesamt Hb bei einem Material ahnlicher Grossenordnung (Ratte Mensch Kalb Pferd) einen Zusammenhang entsprechend Total Hb = 0 013 Korpergewicht 0 99

Da in 6000 m und vor allem in 7000 m Hohe teilweise das Korpergewicht starker absinkt mag die Korperoberflache als Mass des Stoffwechsels hier mit einem starkeren Unsicherheitsmoment behaftet ein Es muss offen bleiben ob die schlechtere Relation von Gesamt Hb und Blutvolumen zur berechneten Korperoberflache in diesen Hohenstufen damit oder mit anderen Faktoren wie Anderungen des Stoffwechsels alterer Tiere unter Hypoxie zusammenhangt worauf der anhaltende Temperaturabfall solcher Tiere hinweisen mag (FLUCKIGER und VERZAR 1955)

Es ware wunschenswert die allgemeine Gultigkeit des hier gefundenen Zusammenhangs zwischen Gesamt Hb und Blutvolumen zur Korperoberflache in Hypoxie an verschiedenen Tierspezies zu uberprufen Die vorliegenden Daten von Hund (REISSMANN 1951 ROTTA zit von HURTADO et al 1945) und Mensch (HURTADO et al 1945 MERINO 1950 REYNAFARJE LOZANO und VALDIVIESO 1953) zeigen zwar durchweg auf das Korpergewicht bezogen niedrigere Werte als die der Ratte in entsprechender Hohe Ein direkter Vergleich ist aber vor allem deshalb ausgeschlossen weil die O Hb-Dissoziationskurve der Ratte gegenuber der des Menschen oder Hundes rechtsverlagert ist *Damit sinkt die O Hb* Sattigung der Ratte bei gegebenem O_2-Druck wesentlich starker *als die des* Menschen oder Hundes wodurch die Erythropoiese erheblich beeinflusst wird. Die kraftige Stimulation der Erythropoiese des Kaninchens in mittlerer Hohe (1800 m) haben WANG WIRZ und VERZAR (1951) *ebenfalls* auf die rechtsgelagerte O Hb Dissoziationskurve des Kaninchens zuruckgefuhrt

1 Nach dem bisher Besprochenen kann angenommen werden dass in Hypoxie die O_2-Versorgung bzw der O Druck des Gewebes entscheidend fur die regulatorischen Vorgange der Erythropoiese ist Damit sollte zwischen

wie hoch unter verschiedenen Bedingungen der O_2 Druck der Gewebe ist Was unter »adaquate O_2 Versorgung des Gewebes« verstanden werden kann wird unten ausfuhrlicher besprochen

Es seien die Anderungen der Erythropoiese unter Hypoxie betrachtet Dabei lassen sich zwei verschiedene Reaktionsformen erkennen Einmal wird der mit Hypoxie abfallende arterielle O_2 Gehalt durch eine erhöhte O_2 Kapazitat des Blutes ausgeglichen Von dieser Anpassungsweise macht der Organismus bis zu 4000 m Hohe fast ausschliesslich Gebrauch Bis zu 6000 m Hohe steigt neben der O_2 Kapazitat das Volumen des Gefassystems fur dessen Grosse das Blutvolumen ein Mass bildet und wobei die Kapillarisation entscheidende Bedeutung hat Bei weiter steigender Hypoxie vergrossert sich schliesslich nur noch das Gefassystem Wie unten naher ausgefuhrt wird erfolgt der Anstieg des Blutvolumens gleichzeitig mit wesentlichen Umstellungen des Kreislaufs Diese Kreislaufumstellung bei Unterschreiten eines gewissen O_2 Drucks im Gewebe durfte durch eine Gefassdilatation ausgelöst werden Die Erythropoiese wird durch diesen Prozess in sofern stark beteiligt als sie nun fur ein vergrossertes Gesamt Blutvolumen zu sorgen hat dessen O_2 Gehalt obendrein noch weiter absinkt

Bei Hohen bis zu 1000 m kann der O_2 Gehalt hoher als normal sein Das spricht dafur dass es nicht einzig darum geht einen konstanten arteriellen O_2 Gehalt aufrecht zu halten Darauf deuten auch die Befunde in extremer Hypoxie hin Beim Übergang von 6000 m auf 7000 m Hohe fallt namlich der arterielle O_2 Gehalt und bleibt auf einem niedrigeren Niveau Ware der erniedrigte arterielle O_2 Gehalt alleinige Ursache der gesteigerten Erythropoiese so sollte in 7000 m Hohe immer mehr Hb gebildet werden Tatsachlich stellen sich aber auch hier Gesamt Hb und Blutvolumen auf neue Niveaus ein und erst in 8000 m Hohe steigt die Hb Bildung erneut stark an Ein Versagen der Erythropoiese in 7000 m Hohe ist daher nicht wahrscheinlich vielmehr ist zu vermuten, dass andere Mechanismen wie die gesteigerte Kapillarisation oder Durchblutung den O_2 Bedarf des Gewebes befriedigen Ahnliche Mechanismen mussen aber auch bereits in 5000 m und 6000 m Hohe wirksam sein da sonst der arterielle O Gehalt wesentlich hoher als normal sein sollte um den unter Hypoxie absinkenden O_2 Druck des Gewebes auf einem unveranderten Niveau zu halten

Der Nachweis dass die Hohe des O_2 Bedarfs des Gewebes auf die Erythropoiese einwirkt kann als weitere starke Stutze dessen angesehen werden dass die Veranderungen der Erythropoiese tatsachlich von der adaquaten O Versorgung des Gewebes abhangig sind Die hier beobachtete gute Korrelation des Gesamt Hb bzw Blutvolumens zur Korperoberflache d h einem indirekten Mass des Stoffwechsels in Hypoxie ist zwar nicht beweisend mag

ähnliche Verhältnisse vorliegen. Dem widersprechen auch Untersuchungen des Knochenmarks colchicinvergifteter Ratten in Hypoxie und Anämie, nach denen an allen teilungsfähigen und vor allem reiferen Zellelementen die Zellproliferation einsetzt (MÖLLER 1960). Erklärt man andererseits diese Latenz mit dem durch die Blutentnahme verbundenen Operationstrauma, ist es ebenfalls nicht zu verstehen, warum sich bei akuter schwerer Hypoxie, die die Tiere vielfach an die Grenze der Lebensfähigkeit bringt, nicht eine ebensolche oder längere Latenz findet. Eine Lösung dieser Frage kann darin zu suchen sein, dass der eigentliche erythropoietische Reiz bei Anämie erst dann auftritt, wenn die Hb-Konzentration des Blutes durch den kompensatorischen Anstieg des Plasmavolumens abfällt. In der Literatur werden je nach Tierspezies, Umfang der Blutung und sonstigen Bedingungen sehr verschiedene Zeitintervalle dafür angegeben. In den eigenen Versuchen wurden teils nach 24 Stunden normale Blutvolumina (Arbeit IV), teils bei etwas kräftigerem Blutverlust nach 48 Stunden noch erniedrigte Blutvolumina gefunden (Arbeit III). Weiterhin ist denkbar, dass je nach der erythropoietischen Reizstärke andere störende Faktoren mehr oder minder zur Geltung kommen. Daraus einen prinzipiellen Unterschied für Anämie und Hypoxie als Reizform der Erythropoiese ableiten zu wollen, erscheint jedoch nicht begründet. Die gefundene normale Lebenslänge unter Anämie gebildeter Erythrozyten stützt ebenfalls nicht die Vorstellung einer Sonderstellung der Blutbildung bei Anämie gegenüber Normalverhältnissen und Hypoxie. Das schliesst aber nicht aus, dass bei anhaltenden und schweren Anämien anatomisch und funktionell abnorme Zellen gebildet werden können (AMBS 1963).

Cobalt erweist sich im Vergleich zu Hypoxie und Anämie als sehr viel schwächerer erythropoietischer Reiz. Die von JACOBSON und GOLDWASSER (1958) beschriebene starke erythropoietinbildende Potenz von Cobalt findet demnach nicht einen entsprechenden Ausdruck in den Blutwerten selbst. Es mag deshalb ratsam erscheinen, nicht allzu weitgehende Schlussfolgerungen aus dem Auftreten derartiger Substanzen für das Regulationsgeschehen der Erythropoiese zu ziehen, zumal auch der als biologischer Standard von WHITE et al. (1960) verwendete erythropoiesestimulierende Effekt von 5 µM $CoCl_2$ von KEIGHLEY (1962) nur bei fastenden Ratten eines bestimmten Stammes und Geschlechts gefunden werden konnte. Nach den Untersuchungen von LEVY, LEVISON und SCHADE (1950) soll die Wirkung von Cobalt über die Hemmung gewisser, auch an der Zellatmung beteiligter Enzyme erfolgen.

5. Mit der Annahme, dass Änderungen der Erythropoiese von der adäquaten O_2-Versorgung des Gewebes abhängen, tritt die Frage auf, in welchem Verhältnis die verschiedenen Faktoren, die auf den O_2-Druck des

Anämie und Hypoxie als Reiz der Erythropoiese kein prinzipieller Unterschied bestehen. Es ist ebenfalls nicht möglich, bei Hypoxie in einem erniedrigten arteriellen O_2-Druck oder O_2-Gehalt für sich die auslösende Ursache einer erhöhten Erythropoiese zu suchen. Man kann lediglich die jeweilige Bedeutung dieser beiden Komponenten für den O_2-Druck des Gewebes versuchen abzuschätzen.

Dem nachfolgenden Vergleich der Wirkung von Anämie mit Hypoxie und Cobalt auf die Erythropoiese sei eine sehr grobe Überschlagsrechnung des mittleren venösen und mittleren kapillaren O_2-Drucks bei Hypoxie und Anämie vorausgestellt. Der venöse O_2-Druck wurde unter der Annahme einer arterio-venösen O_2-Differenz von 5 Vol.% aus dem arteriellen O_2-Druck, der arteriellen O_2Hb-Sättigung und der von JONES, MACGRAITH und SCULTHORPE (1950) angegebenen (leicht veränderten) O_2Hb-Dissoziationskurve der Ratte berechnet. In Hypoxie wurde die Linksverlagerung der Kurve mit dem verminderten CO_2-Druck in Rechnung gestellt, nicht jedoch die in ihrem Umfang hier nicht bekannte Rechtsverlagerung der Kurve bei Anämie. Für den mittleren kapillaren O_2-Druck ($P_{\bar{c}O_2}$) gilt nach BARCROFT (1931) approximativ $P_{\bar{c}O_2} = P_{\bar{v}O_2} + \frac{P_{aO_2} - P_{\bar{v}O_2}}{3}$; $P_{\bar{v}O_2}$, P_{aO_2} = mittlerer venöser bzw. arterieller O_2-Druck. Danach beträgt unter Normalbedingungen bei einem arteriellen O_2-Gehalt von 15 Vol.% der mittlere venöse bzw. kapillare O_2-Druck 56 bzw. 67 mm Hg. In 4000 m und 6000 m Höhe sinkt die arterielle O_2Hb-Sättigung auf 65% und 56%, der arterielle O_2-Gehalt auf 10,7 Vol.% und 9,2 Vol.%, der mittlere venöse O_2-Druck auf 32 und 24 mm Hg, der mittlere kapillare O_2-Druck auf 38 und 28 mm Hg. Eine gleiche Senkung des arteriellen O_2-Gehalts durch Anämie führt zu einem venösen O_2-Druck von 47 und 42 mm Hg und zu einem mittleren kapillaren O_2-Druck von 61 und 58 mm Hg.

Vergleicht man auf der Basis dieser mehr qualitativen als quantitativen Berechnungen die Hb-Bildung bei Anämie und Hypoxie, so ist die sehr viel geringere Bildungsleistung bei Anämie leicht verständlich. Die Hb-Bildung bei Anämie wird auf ihrer Höhe etwa verdoppelt, in Hypoxie bei etwa gleich niedrigem arteriellen O_2-Gehalt etwa vervierfacht. Einige qualitative Unterschiede im Hb-Bildungsverlauf sind demgegenüber nicht ohne weiteres verständlich. Bis zum Hb-Anstieg nach Beginn der Anämie vergehen etwa 72 Stunden, in Hypoxie werden bereits nach 18 Stunden maximale Bildungswerte gefunden. Erklärt man diese Latenz bei Anämie mit ERSLEV (1959) damit, dass der erythropoietische Reiz an den Stammzellen angreift und eine solche Zeit vergeht, bis für eine gesteigerte Hb-Synthese die notwendigen Zellen zur Verfügung stehen, so bleibt unklar, warum in Hypoxie nicht

ähnliche Verhältnisse vorliegen Dem widersprechen auch Untersuchungen des Knochenmarks colchicinvergifteter Ratten in Hypoxie und Anämie nach denen an allen teilungsfähigen und vor allem reiferen Zellelementen die Zellproliferation einsetzt (MOLLER 1960) Erklärt man andererseits diese Latenz mit dem durch die Blutentnahme verbundenen Operationstrauma so ist ebenfalls nicht zu verstehen warum sich bei akuter schwerer Hypoxie die die Tiere vielfach an die Grenze der Lebensfähigkeit bringt nicht eine ebensolche oder längere Latenz findet Eine Lösung dieser Frage kann darin zu suchen sein dass der eigentliche erythropoietische Reiz bei Anämie erst dann auftritt wenn die Hb Konzentration des Blutes durch den kompensatorischen Anstieg des Plasmavolumens abfällt In der Literatur werden je nach Tierspezies Umfang der Blutung und sonstigen Bedingungen sehr verschiedene Zeitintervalle dafür angegeben In den eigenen Versuchen wurden teils nach 24 Stunden normale Blutvolumina (Arbeit IV) teils bei etwas kräftigerem Blutverlust nach 48 Stunden noch erniedrigte Blutvolumina gefunden (Arbeit III) Weiterhin ist denkbar dass je nach der erythropoietischen Reizstärke andere störende Faktoren mehr oder minder zur Geltung kommen Daraus einen prinzipiellen Unterschied für Anämie und Hypoxie als Reizform der Erythropoiese ableiten zu wollen erscheint jedoch nicht begründet Die gefundene normale Lebenslänge unter Anämie gebildeter Erythrozyten stützt ebenfalls nicht die Vorstellung einer Sonderstellung der Blutbildung bei Anämie gegenüber Normalverhältnissen und Hypoxie Das schliesst aber nicht aus dass bei anhaltenden und schweren Anämien anatomisch und funktionell abnorme Zellen gebildet werden können (AMBS 1963)

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Gewebes einwirken zueinander stehen Dabei handelt es sich im wesentlichen um folgende Grossen Durchblutung O Gehalt des *Blutes interkapillarer* Abstand kapillare Diffusionsoberflache sowie O_2 Verbrauch des Gewebes Die Bedeutung einiger dieser Grossen fur den O_2 Geweb druck ist von KETY (1957) abgeschatzt worden Der fur einen gewissen Punkt eines Normal gewebes berechnete O_2 Druck von 32 mm Hg steigt danach um *etwa 10—15 mm Hg*, wenn entweder die Durchblutung um 50 % steigt oder der halbe interkapillare Abstand um 50 % sinkt Steigt der O_2 Verbrauch um 50 % *sinkt umgekehrt der O_2 Druck um etwa 20 mm Hg* Die Veranderungen des O_2 Gehalts sind etwa denen einer Durchblutungsanderung vergleichbar Sie sind allerdings *nach oben hin stark be chrankt Hinzu kommt*, dass die Viscositat bei Überschreiten eines Hamatokrits von etwa 60 % stärker ansteigt (GREEN 1950), die Verhaltnisse sind aber schwer zu überblicken da gleichzeitig auch der Kapillardiameter anzusteigen scheint (OPITZ 1952)

Ein intimer Zusammenhang zwischen der Hohe der Kapillarisation und den Anderungen im O_2 Gehalt des Blutes als Mittel, auf den O_2 Druck des Gewebes einzuwirken kann wie oben und auch in Arbeit X be prochen worden ist vermutet werden *Hier soll auf die Bedingungen* eingegangen werden, unter denen der O_2 Bedarf des Gewebes uberwiegend durch Durch*blutungsanderungen bzw Anderungen in der O Kapazitat des Blutes befriedigt* wird

In Meereshohe wird ein gesteigerter O_2 Bedarf im wesentlichen gänzlich durch eine erhöhte Durchblutung gedeckt Weder andert haufig ausgeubte Muskelarbeit die O_2 Kapazitat des Blutes noch kann der verhaltnismä ig sehr bescheidene Effekt der Thyreoidea auf die Erythropoiese als spezifi ch erythropoietisch angesprochen werden (GRANT und ROOT 1952 VAN DIKE et al 1954 GEMZELL et al 1963) Auch bei O_2 Mangel kommt es zu Durch blutungssteigerungen Charakteristi ch fur diese ist dass sie in sehr gute *Relation zum venosen O Druck gestellt werden konnen* (NOELL und SCHNEIDER 1942 NOELL 1944 — Gehirndurchblutung KORNER 1959 — Herzminutenvolumen) Dabei i t gleichgultig, ob arterielle Hypoxie Anämie Oligamie oder CO Vergiftung vorliegt und um welche Tierspezies es sich handelt (Mensch Hund Kaninchen) Die Durchblutung steigerung setzt bei einem venosen O Druck von etwa 35—30 mm *Hg* ein entsprechend einem etwa in 1000 m Hohe herrschenden arteriellen O Druck oder einem Anamie grad von etwa 7 g % *Hb Dieser O Druck stellt auch in den vorliegenden* Untersuchungen einen Grenzwert dar bei dem das Blutvolumen anzusteigen beginnt Fur die Zirkulation *in Hypoxie ist ferner bemerkenswert* dass die gesteigerte Pulsfrequenz etwa 1 Woche nach einsetztender Hypoxie wieder auf den Ausgangswert abzusinken pflegt (KORNER 1959) Das Herzminuten

6 Ein vielerörtertes Problem ist, wo der O_2 bzw O_2-Mangel in die Regulation der Erythropoiese eingreift Ein direkter Einfluss auf das *Knochenmark* wird jetzt allgemein abgelehnt Die Gründe dafür haben GRANT und ROOT (1952) ausführlich besprochen O_2 wirkt demnach indirekt auf die Erythropoiese über im Gewebe gebildete Zwischenprodukte sogenannte Hämatopoietine (CARNOT und DEFLANDRE 1906) oder Erythropoietine (BONSDORFF und JALAVISTO 1948), ein

Es ist bisher nicht sicher geklärt, welche Gewebe unter O_2-Mangel diese Erythropoietine bilden Die verschiedensten endokrinen Organe *Nebennieren Thyreoidea, Keimdrüsen* und *Hypophyse* haben zwar einen gewissen modifizierenden Einfluss auf die Erythropoiese *an der prinzipiellen hypoxischen* Reaktion eines Organimus ändern sie jedoch nichts (VAN DYKE et al 1954 PILIERO 1959) Auch der *Hypothalamus* der mit der endokrinen Regulation eng verbunden ist hat keine Bedeutung für die Entwicklung der Polyzythämie bei Hypoxie (PILIERO MEDICI und ORR 1962)

Die von STOHLMAN, RATH und ROSE (1953 1954) sowie SCHMID und GILBERTSEN (1955) beschriebene *kräftige* Polyzythämie *bei* 2 Fällen mit offenstehendem Ductus arteriosus *mit* hohem Druck im Lungenkreislauf wodurch die obere Körperhälfte mit *normal* O_2 gesättigtem die untere Körperhälfte mit stark vermindert O_2 gesättigtem Blut versorgt wird zeigt dass das *Gehirn* nicht der primäre Angriffspunkt von Hypoxie ist Das in einem Fall hypoplastische Knochenmark der unteren Körperhälfte *und* das hyperplastische Knochenmark der oberen Körperhälfte bei beiden *Fällen demonstriert* weiterhin, dass der herabgesetzte O Druck nicht primär das Knochenmark stimuliert

Meerschweinchen ohne *Milz* reagieren auf Hypoxie ähnlich wie Normaltiere (GORDON *und* KLEINBERG 1937)

Die reichlichen Untersuchungen über die Bedeutung der *Niere* ergeben kein eindeutiges Bild (Für *eine* eingehende Diskussion s *zusammenfassende* Übersichten) Das beruht u a darauf *dass nephrektomierte* Tiere *nur kurze* Zeit überleben bzw durch die sich entwickelnde Urämie stark beeinträchtigt werden Vielfach *sind indirekte Tests herangezogen* worden, wobei der erythropoiese stimulierende Effekt von *Plasma nephrektomierter oder* ureterunterbundener Tiere an hypophysektomierten, *hungernden oder* polyzythämischen *Tieren* geprüft worden ist In Hypoxie haben GOLDWASSER *et al* (1958) keinen gesteigerten erythropoietischen Effekt im Plasma nephrektomierter Tiere im Gegensatz zu ureter *unterbundenen oder normalen* Tieren gefunden MIRAND PRENTICE und SLAUNWHITE (1959) *fanden demgegenüber auch beim nephrektomierten Tier in* Hypoxie einen vollen Effekt LANGE und GALLAGHER (1962) einen *abgeschwächten In Parabiose*versuchen bei denen sich der

eine Hypoxiehypothese gegenübergestellt (OPITZ 1918). Nach den Berechnungen des O_2-Drucks des Gehirns von OPITZ und SCHNEIDER (1950) schien für die Reaktionen dieses Organs die Hypoxiehypothese wahrscheinlicher. Neuere Untersuchungen von HIRSCH et al. (1955) und Berechnungen von THEWS (1960) mögen eher die Anoxiehypothese stützen. Ein wesentlicher Fortschritt zur Klärung dieser Frage sind vor allem *in vivo*-Messungen des Oxydationszustands der Pyridin-Nucleotide. CHANCE, COHEN und SCHOENER (1962) fanden einen beginnenden Anstieg des Reduktionszustandes der Pyridin-Nucleotide der Hirnrinde der Ratte bei einer 8%igen O_2-Konzentration der Einatmungsluft entsprechend einem intrazellulären O_2-Druck von etwa 1 mm Hg.

Es ist nicht möglich, sich definitiv dazu zu äussern, ob die Regulation der Erythropoiese entsprechend der Anoxiehypothese oder Hypoxiehypothese vor sich geht. Wie das Absinken der Erythropoiese bei hohem O_2-Gehalt des arteriellen Blutes zeigt, z.B. beim Übergang eines in Höhe lebenden Organismus auf Meereshöhe, besteht bereits bei Luftatmung ein vom O_2-Druck abhängiger Bildungsreiz. Nach der Anoxiehypothese müsste also bereits hier die Atmungsgeschwindigkeit gewisser Zellen, von denen dieser Reiz ausgeht, eingeschränkt sein. Die Hypoxiehypothese ermöglicht es, nicht nur den erythropoietischen Reiz bei normalem O_2-Druck, sondern auch in mittleren Hypoxiegraden, bei dem die Gesamt-O_2-Aufnahme offenbar noch keine messbare Einschränkung erfährt, zu erklären. Ob der fehlende erythropoietische Reiz bei gesteigertem O_2-Bedarf des Gewebes und Atmung in Normalluft mit dem dabei im wesentlichen unveränderten Oxydationszustand zumindest der terminalen Oxydase der Atmungskette zusammenhängt (CHANCE 1957), muss ebenfalls offen bleiben.

Zusammenfassung und Schlussfolgerung

Der vorliegende Beitrag zur Frage der Regulation der Erythropoiese wurde besonders im Hinblick auf die funktionelle Bedeutung des Hämoglobins als O_2-Träger und die O_2-Versorgung des Gewebes ausgeführt. Dabei wurden die Änderungen der gesamten Hämoglobinmenge, der relativen Blutwerte und des Blutvolumens der Ratte bei vielwöchentlichem Aufenthalt in O_2-Drucken zwischen 760 und 50 mm Hg, bei künstlich verändertem O_2-Gehalt des Blutes und unter Cobalt-Zufuhr untersucht.

Im ersten methodologischen Teil wird ein Verfahren beschrieben, mit dessen Hilfe unter Verwendung von CO beliebig oft und vom selben Tier die gesamte Hämoglobin-Menge bestimmt und verfolgt werden kann. An

Mangel Hb zu bilden beginnt (Fox und PHEAR 1953) Es ist denkbar, dass der Stimulation der Hb Bildung durch O_2 Mangel bei Saugern und niedrigen Tieren gleiche biochemische Prozesse zugrunde liegen

7 Neben der Frage, wo der O_2 in die Regulation der Erythropoiese ein greift interessiert in hohem Grade wie das geschieht — wahrscheinlich der Kernpunkt des Regulationsproblems der Erythropoiese Dabei sind zwar Folgeerscheinungen von O_2 Mangel bekannt beispielsweise das Auftreten von erythropoiese stimulierenden Substanzen Die primaren, mit dem O_2 ver bundenen Prozesse sind aber weder bekannt, noch bestehen klare Vorstellungen daruber wie sie aussehen konnten

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Der vorliegende Beitrag zur Frage der Regulation der Erythropoiese wurde besonders im Hinblick auf die funktionelle Bedeutung des Hämoglobin als O-Träger und die O-Versorgung des Gewebes ausgeführt Dabei wurden die Änderungen der gesamten Hämoglobinmenge der relativen Blutwerte und des Blutvolumens der Ratte bei vielwöchentlichem Aufenthalt in O Drucken zwischen 760 und 50 mm Hg bei künstlich verändertem O-Gehalt des Blutes und unter Cobalt Zufuhr untersucht

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Hand von Vergleichsuntersuchungen der Erythrozytenmenge mit radioaktiv gezeichneten Erythrozyten, Messungen der Hämoglobin-Menge vor und nach Entnahme einer bekannten Menge Hämoglobin sowie Erfahrungen nach mehrjährigem Gebrauch werden Fehlermöglichkeiten und Anwendbarkeit der Methode diskutiert. Eine entwickelte Mikromethode zur Isolierung von Hämin aus kleinen Blutmengen erlaubt es, neben der spezifischen Aktivität der Häminkomponente radioaktiv *in vivo* gezeichneten Hämoglobins zusammen mit diesem Verfahren die gesamte Häminaktivität eines Tieres zu verfolgen. Unabhängig von Verdünnungseffekten durch neugebildetes Hämoglobin wurde so die Erythrozytenlebenslänge nach akuter Blutung neugebildeter Erythrozyten bestimmt. Diese kombinierte Methode wurde auch zum Grobnachweis hämolytischer Reaktionen herangezogen.

Im zweiten Teil werden die Untersuchungsergebnisse zusammenfassend dargestellt. Anämie, Hypoxie und Cobalt als Reizformen der Erythropoiese werden miteinander verglichen. Die Resultate werden vor allem in Hinblick auf die Bedeutung des Sauerstoff für die Regulation der Erythropoiese unter Berücksichtigung anderer Untersuchungen und der Vorstellungen dazu besprochen.

Als entscheidend für alle regulatorischen Veränderungen der Erythropoiese kann die Höhe des O_2-Drucks des Gewebes angenommen werden. Abweichungen des O_2-Drucks des Gewebes von der Norm führen jedoch nur dann zu einer veränderten Erythropoiese, wenn diese durch herabgesetzten arteriellen O_2-Gehalt bzw. O_2-Druck (Anämie, Hypoxie) oder erhöhten O_2-Gehalt des Blutes (Aufenthalt in Meereshöhe nach Höhenakklimatisation) hervorgerufen werden. Ein erhöhter O_2-Bedarf des Gewebes wird im wesentlichen durch eine gesteigerte Durchblutung gedeckt. O_2-Mangel scheint nicht direkt auf das Knochenmark, sondern über im Gewebe gebildete erythropoiese-stimulierende Substanzen zu wirken. Die *offenbaren biochemischen und* physiologischen Fragestellungen sind, wie und wo O_2 an der Bildung dieser Substanzen *beteiligt ist. Ein weiteres Problem ist, nach welchen* Regeln O_2-Gehalt des Blutes, Kapillarisation und kapillare Durchblutung an der *O_2-Versorgung des Gewebes teilnehmen.* Bei der Bearbeitung derartiger Fragen erscheint es zweckmässig, den O_2-Gehalt des Blutes als ein Glied *im Prozess* der O_2-Versorgung des Gewebes und nicht im Sinne einer homöostatisch regulierten gegebenen Grösse zu betrachten.

Die Arbeit wurde auf Anregung und Einladung Professor TORGNY SJÖSTRANDS Klinisch physiologische Abteilung des Karolinska Sjukhuset Stockholm ausgeführt Ich möchte Herrn Professor SJÖSTRAND der diese Untersuchungen nicht nur grosszügig finanziell ermöglicht hat, sondern mich bei allen Problemen und Fragen freundschaftlichst beraten und unterstützt hat meinen herzlichen Dank zum Ausdruck bringen

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PULMONARY GAS EXCHANGE IN MAN AS AFFECTED BY PROLONGED GRAVITATIONAL STRESS

BY

PER OLOF BARR

STOCKHOLM 1963

CONTENTS

Stockholm 1963
Kungl Boktryckeriet P A Norstedt & Söner

CONTENTS

I PREFACE

The present work is part of a research program concerning the pulmonary gas exchange as affected by environmental stress factors

It is a great pleasure for me to extend my heartfelt thanks to Dr H Bjurstedt who has devoted much of his time to introducing me to the essentials of the work and with whom I have had many fruitful and stimulating discussions

To Professor U S von Euler the pioneer of experimental aeromedicine in Sweden, I wish to pay special tribute for constant support and encouragement throughout my education in this field

To Dr C M Hesser I should like to express my great appreciation for stimulating advice as to the design of the experimental procedures

My thanks are also due to Dr G Rosenhamer for reliable and attentive assistance in the processing and presentation of experimental data

For the excellent assistance they provided throughout the experimental work I am much indebted to the entire technical staff of this laboratory

This investigation was supported in part by a research contract with the Wright Air Development Division of the Air Research and Development Command United States Air Force through its European Office

Stockholm April 1963

PER OLOF BARR

logical significance discussed A commentary is included on certain applications of the results to problems involved in operator performance and flight safety

List of Symbols and Abbreviations

Primary Symbols

- V Gas volume in general
- V̇ Gas volume per unit time
- Q̇ Volume flow of blood
- P Pressure in general including partial pressure
- F Fractional concentration in dry gas phase
- C Concentration in blood phase
- S Oxygen saturation of hemoglobin per cent

Suffixes

Gas phase

- I Inspired gas
- E Expired gas
- T Tidal gas
- A Alveolar gas
- D Dead space

Blood phase

- a Arterial
- $\bar{v}$ Mixed venous

Other Symbols

- R Respiratory exchange ratio (volume CO_2/volume O_2)
- STPD Standard temperature and pressure dry (0 C 760 mm Hg)
- BTPS Body temperature and pressure saturated with water vapor
- ATPS Ambient temperature and pressure saturated with water vapor

For definitions and terminology concerning accelerative and gravitational forces with respect to magnitude direction and duration the principles used by GAUER (1961) have been adopted

For the statistical treatment of data current conventions have been used

- n Number of observations
- Range Smallest and greatest observed value
- $\bar{x}$ Arithmetic mean
- $\varepsilon\bar{x}$ Standard error of the mean
- p Probability

The statistical significance of differences between means was evaluated by applying the t test to the *individual* differences (*cf* FISHER 1948) $p < 001$, $p < 01$ $p < 05$ denote highly *significant* significant and probably significant differences respectively

II INTRODUCTION

By exposing the body to forces which are qualitatively equivalent to but considerably stronger than those caused by normal gravity, it is possible to exaggerate the physiological responses that would normally occur when body posture is changed from lying to sitting or standing Such forces can readily be produced in the laboratory by means of the large centrifuges that are currently being used in aerospace medical research

The present investigation is an experimental study of certain effects of prolonged gravitational stress on the principal function of the pulmonary circulation, *i e* the exchange of O_2 and CO between the alveolar spaces and the blood Earlier results from centrifuge studies in this laboratory on animals and on man, have demonstrated that inertial forces may lead to a marked fall in the arterial O saturation in the face of a considerable increase in the pulmonary ventilation, indicating the development of a large venous admixture (physiological shunt) to the arterialized blood The present experiments were performed exclusively on human subjects While exposed to strong gravitational fields for periods up to 2 minutes with the G force acting in the head to foot direction, the subjects displayed drastic alterations in the pulmonary gas exchange as revealed by continuous and simultaneous recordings of ventilatory and blood chemical variables In this way some of the mechanisms which normally influence alveolar ventilation and pulmonary blood flow, independently and in relation to each other both throughout the lungs and regionally, could be studied by the relatively simple approach of exaggerating the influence exerted by normal gravity

It became evident that the pulmonary circulation is highly susceptible to inertial forces there is probably no influence, environmental or of other origin that is capable of exerting a reversible effect on pulmonary function which is so profound The extent to which such forces were found to affect the pulmonary gas exchange in standardized exposures was investigated on the basis of relatively recent concepts pertaining to the ventilation perfusion relationships of the lung

In the organization of the text it seemed appropriate first to give a short review of earlier findings pertaining to the effects of inertial forces on the pulmonary circulation and gas exchange The review serves as a background to the problems of the present work which are defined mainly in terms of their fundamental physiological nature In view of the special and sometimes adverse experimental conditions involved in the methodological approach to the problems, a relatively comprehensive account of the techniques and experimental procedure is given before the results are described and their physio

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III BACKGROUND

There is theoretical as well as experimental evidence that the pulmonary circulation and gas exchange are susceptible to the stress of normal gravity, and to the qualitatively equivalent but stronger forces of inertia which occur during flight in high performance aircraft and space vehicles One may *a priori* expect that the increased 'effective' weight of the blood would be potentially capable of producing gross disturbances in the pulmonary circulation and thus become detrimental to the main function of the lung *i e* that of exchange of O and CO_2 between the blood and the ambient air This becomes clear from the simple reasoning that, with an increasing weight of the blood, a condition would eventually be reached in which all the blood in the lung would go to its 'dependent" regions (no ventilation), and all ventilation would go to other regions (no perfusion) Thus even if the total ventilation and blood flow through the lungs were normal, no gas exchange would be possible and asphyxia would rapidly develop

In the following a brief review is given of some earlier investigations pertaining to the influence of the inertial forces on the pulmonary circulation and gas exchange

Effects of Gravitational Forces in the Head to Foot Direction

Effects of normal gravity MARTIN, CLINE and MARSHALL (1953) observed by sampling alveolar gas simultaneously in man's right upper and lower lobes that in the upright position the CO_2/O_2 ratio was larger in the upper lobe, while in the supine and the head down position the ratio was the same In later experiments the same investigators (1955) obtained evidence that these differences could be referred to a larger alveolar ventilation perfusion ratio in the upper lobe while standing These findings were of considerable interest since they gave the first indication by direct sampling that the alveolar gas composition is not necessarily uniform in different lobes Thus a difference in ventilation perfusion ratio was established but the lack of direct measurements of lobar ventilation did not allow predictions as to whether the upper lobe is relatively overventilated or underperfused

The observations of INADA *et al* (1954) who used differential spirometry for measurements of O_2 uptake and ventilation of each lung in the left and right lateral positions, made it likely that in either lateral position propor

tionally more blood than ventilation is shifted to the dependent lobe MATTSON and CARLENS (1955) using a similar approach demonstrated a shift in blood flow from the upper to the lower lobe when the position of the subject was changed from supine to standing Together these studies allowed the conclusion that normal gravity can shift the ventilation perfusion ratios in the subdivisions in the lung That a shift from the supine to the erect posture produces apical underperfusion rather than overventilation has later been confirmed by other investigators (MARTIN and YOUNG 1957 RILEY *et al* 1959 WEST and DOLLERY 1960 DOLLERY DYSON and SINCLAIR 1960)

The efficiency of pulmonary gas exchange in the erect posture can also be assessed by direct measurements of O and CO_2 tension differences between mean alveolar gas and arterial blood BJURSTEDT *et al* (1962) who based their measurements on continuous and simultaneous recordings of arterial O_2 saturation and pH and on breath by breath recordings of end tidal CO_2 tension observed a significant rise in the arterial to end tidal CO tension difference (average + 2 1 mm Hg) in human subjects when body position was changed from supine to standing A CO_2 difference of the same order of magnitude was obtained by ULMER and REICHEL (1961) BJURSTEDT *et al* also observed an increased effective alveolar to arterial O_2 tension difference (average + 4 8 mm Hg) in the erect posture

Effects of hypergravitational stress The above mentioned investigations clearly indicate that in the erect position man s pulmonary vasculature is unable to compensate completely for the normal acceleration of gravity Hyper gravitational stress as produced in various flight maneuvers and during centrifugation exaggerates normally encountered gravitational effects on the pulmonary circulation This was first observed in X ray motion picture studies (monkey) in which contrast material was slowly infused (GAUER 1944 reviewed by GAUER and BONDURANT 1961) It could be observed that at accelerations higher than + 2 G the contrast material was carried only into the pulmonary region below the heart Similar conclusions can be drawn from X ray pictures in man (VON DIRINGSHOFEN 1942 HERSHGOLD 1960)

That desaturation of arterial blood may develop during headward acceleration was first reported briefly by GAUER *et al* (1949) HENRY (1950) and HENRY *et al* (1951) The implications of these observations were studied in anesthetized dogs by BARR BJURSTEDT and COLERIDGE (1959 b) who recorded respiration arterial O_2 saturation and pH under headward acceleration It was observed that application of moderate G forces over several minutes produced severe hypoxemia although 100 % O_2 was breathed and hyper ventilation was present indicating a great alveolar arterial O_2 difference and accordingly a large intrapulmonary shunt BARR (1962) extended these studies to man under headward acceleration In these experiments the arterial O_2 saturation was recorded by continuous cuvette oximetry before during and after prolonged exposures to headward acceleration (4 to 5 G) With the subjects

breathing air and wearing an automatically inflated anti G suit the arterial O saturation decreased within the first minute to between 95 and 81 per cent, the arterial pH remaining essentially unchanged although respiratory minute volume was greatly increased The rate of resaturation on returning to normal gravity was usually slow, especially after repeated exposures The arterial hypoxemia was interpreted as being mainly a shunt effect, caused by congestion with collapse of alveoli ("atelectasis") in dependent regions of the lung

It has been reported (ERNSTING 1960) that when aviators breathing 100 % oxygen and equipped with anti G suits were exposed to headward acceleration in fighter aircraft specific symptoms from the respiratory tract often occurred The main complaints were substernal pain, coughing attacks and difficulties in breathing The origin of these effects, at first obscure, was judged from X ray pictures to be congestion of blood with collapse of alveoli in dependent parts of the lung These changes were not due to effects from O_2 inhalation alone since virtually no symptoms appeared when the aviators were not subjected to high positive accelerations

G Forces in the Chest to Back Direction

Due to the rapid development in aerospace technology during the last few years, interest has been focussed on respiratory effects of forward acceleration ("astronaut position") The end point of tolerance to *G forces acting in the direction* chest to back is determined by respiratory difficulties (*cf* CHERNIACK, HYDE and ZECHMAN 1959) and not by failure of cerebral circulation The respiratory disturbances under forward acceleration have also been investigated by ZECHMAN CHERNIACK and HYDE (1960) WATSON CHERNIACK and ZECHMAN (1960), STEINER, MUELLER and CHERNIACK (1961), and STEINER and MUELLER (1961) whereas the advantages of reversing the direction of the G force ("backward" acceleration) have been described by ROGERS and SMEDAL (1961) and SMEDAL *et al* (1963)

The "shunt effect", explaining part of the deterioration of pulmonary function during forward acceleration has been investigated by NOLAN *et al* (1961) and by STEINER and MUELLER (1961) WOOD *et al* (1961) who were the first to report a marked decrease in the arterial O_2 saturation during forward acceleration recorded a fall below 85 per cent at 5 G by use of cuvette oximetry STEINER and MUELLER reported a decrease to 71 per cent at 8 G with incomplete recovery after 3 min unless O_2 therapy was used The last mentioned investigators interpreted their findings as a result of alteration in ventilation to blood flow ratios throughout the lung with approximately 50 per cent of the cardiac output shunted through totally non ventilated areas at 8 G

IV PROBLEMS

The effects of G forces on pulmonary circulation and gas exchange which were reviewed in the preceding section assume a high degree of significance in relation to the potential hazards of prolonged acceleration in modern high performance aircraft Visual blackout and loss of consciousness as experienced in fighter aircraft are well known effects of short lasting exposures to headward acceleration until recently concepts of the cardiovascular basis of these phenomena have largely been limited to considerations of systemic circulatory effects alone Currently the increasing flight speeds and the much longer duration of exposures to G stress have shifted the emphasis from the gross end points of blackout and unconsciousness to the more subtle but increasingly important decline in human performance which may result from acceleration lasting several minutes

It is therefore important that an understanding be gained of the physiological basis of deterioration in performance under G stress of long duration and of magnitudes below those causing blackout or unconsciousness Although marked arterial O_2 desaturation is known to occur during prolonged acceleration quantitative information of the magnitude of arterial blood gas changes and their time-courses is largely lacking this is especially true in the case of headward acceleration in the conventional seated position *i e* with the G forces acting in the head to foot direction

It appeared that the information required could be obtained by special techniques previously developed for the continuous and simultaneous measurement of arterial O_2 saturation and pH in subjects exposed to accelerative forces on the centrifuge Furthermore it seemed that the same techniques could be profitably applied to the analysis of alterations in the pulmonary gas exchange if combined with methods for the evaluation of alveolar arterial gas tension differences *It was therefore considered worthwhile to study* in a quantitative way the effects of prolonged headward acceleration on alveolar and arterial gas tensions the time courses of these effects and the mechanisms underlying them

Apart from their practical interest such studies seemed to be of significance from a more fundamental point of view in that the effects revealed by exaggerating the effective force might facilitate the understanding of adaptive responses of the body to the stress of normal gravity

V METHODS

Before describing the techniques and procedures used in the present work some general remarks about the experimental approach seem warranted. Previous investigations in this laboratory have demonstrated the advantages of studying the time courses of blood gas changes during exposures to gravitational stress (BARR, BJURSTEDT and COLERIDGE 1959b; BARR 1962), the information obtained by continuous recordings being considerably more complete than would be possible by "spot sampling", because of the speed at which these changes occur. The present investigation involved direct, continuous and simultaneous recordings of changes in the arterial O_2 saturation and pH (see below, "Techniques"). End tidal CO_2 tension was also recorded continuously by use of a breath by breath sampling technique in combination with an infrared CO_2 recording system. Quantitative analyses of the disturbances in pulmonary gas exchange evoked by gravitational stress were obtained from the recordings; conventional analyses of expired gas and of arterial blood samples were also made.

In all experiments the subjects were exposed to a force of five times that exerted by normal gravity (in a few instances the effects of 6 G were tested for comparison) and the force vector was directed along the long axis of the body in the head to foot direction ("positive" G or headward acceleration). The time of exposure was 2 min so that if G were plotted against time one would obtain, for a single experiment, the following G time pattern: (A) a control period under the force of normal gravity (+ 1 G), (B) a rapid rise of the G force to a plateau value of + 5 G lasting for 2 min followed by a rapid return to normal gravity, and (C) a subsequent period of continued observation at normal gravity. As will be described below, the use of counter pressure over the lower half of the body (anti G suit) was required to prevent loss of consciousness during the exposures to increased G stress.

The above mentioned G time pattern was chosen for several reasons. Preliminary observations showed that exposures to forces exceeding + 4 G during air breathing often resulted in marked changes in the arterial O_2 saturation if the anti G suit was activated during centrifuge runs. The rate of fall in the arterial O_2 saturation was such as to generally level off within one minute provided the G level was kept constant over one minute or a somewhat longer period of time. The plateau level of acceleration was therefore maintained for 2 min to permit arterial blood sampling over one minute of relatively stable conditions at the end of the exposure.

The next step in the planning was to decide upon a suitable magnitude of the G force during the exposures. Preliminary experiments showed that rela

tively consistent changes in ventilation and arterial O_2 saturation were obtained if the plateau level of acceleration was kept at + 5 G It was therefore decided to use this level as a standard in the present experiments Another reason for this choice was the desirability of studying the effects of G stress which are often encountered during routine flight missions and which are perhaps more interesting from the viewpoint of flight safety than is the lower stress of + 4 G In consequence it was preferred to use volunteers from an air force wing who were familiar with the stresses of high G levels

Although some of the subjects might have tolerated the standard G time pattern of + 5 G for 2 min without the protection afforded by counter pressure over the lower half of the body, such protection was considered necessary to avoid accidental loss of consciousness and to ensure uniformity in the experimental conditions Accordingly the subjects used anti G suits throughout the experimental series For comparison Fig 5 (p 22) shows some of the hemo-respiratory effects which accompanied exposure to + 5 G in an experiment where the anti G suit was not inflated Because of imminent risk of loss of consciousness the run had to be terminated before 50 sec had elapsed A rather insignificant fall in the arterial O saturation can be seen whereas both the end tidal CO_2 tension and arterial hydrogen ion concentration showed a marked fall

Because of earlier observations (BARR 1962) that repeated exposures to gravitational stress produce residual desaturation of the arterial blood it was considered important to exclude such effects in comparisons of inter individual differences of reaction Therefore the present results refer to the effects obtained in the first run of the day of experiment

The withdrawal of 8 ml blood per *minute from* the radial artery (see p 15) which was required for the continuous *recordings of* arterial O_2 saturation and pH was well tolerated as *judged by the fact* that the heart rate (group mean) at the end *of the* experiment did not differ significantly from the pre-run value (*cf* Table II last column) *It is also worth* mentioning in this connection that MEEHAN and JACOBS (*1959*) did not observe any decrease in the G tolerance in *subjects in which* total blood volume had been reduced to 72 per cent by prolonged bed rest

None of the *subjects* displayed any inconvenience from the indwelling Teflon arterial catheter which *was especially* designed for the present centrifuge experiments Nor did *any* adverse effects result from the administration of heparin which was *necessary* to prevent clotting in the sensing units of the pH-oximeter recording *assembly*

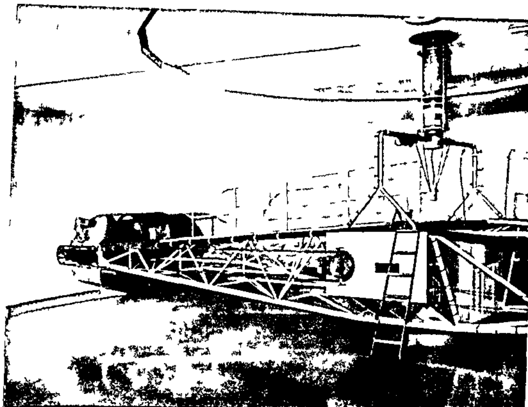


Fig 1 View of gondola and part of the superstructure of the human centrifuge at the Karolinska Institutet

Techniques

Centrifuge

All trials were run in the human centrifuge (Fig 1) at the Karolinska Institutet the subjects were seated in the gondola that is suspended at one end of the double arm superstructure 7 3 m from the center of rotation On starting the centrifuge the gondola swings out so that when a constant speed has been attained its floor remains perpendicular to the direction of the resultant vector For a description of the centrifuge see GOTZLINGER and HELSING (1955)

Measuring and Recording System

A schematic representation of the general experimental set up used in the centrifuge gondola is given in Fig 2 Several recently designed devices were used for the collection of data from the subject in the gondola before during and after centrifugation They were all checked in numerous centrifuge runs and redesigned if needed until free from output variations due to electrical mechanical or other extraneous disturbances during centrifugation

Arterial pH and O_2 saturation were recorded continuously and simultaneously by means of a pH oximeter assembly The physical dimensions dynamic response and electrical circuitry used with this assembly as well as the methods of calibration have been described elsewhere (BARR and BJURSTEDT 1963) The temperature of the blood (about

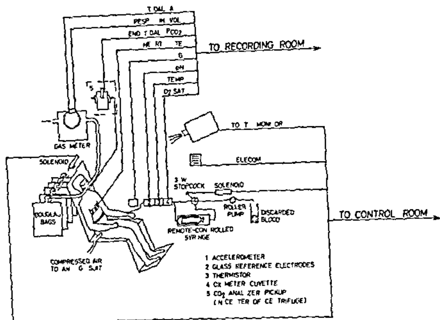


Fig 2 Schematic representation of experimental set up in the centrifuge gondola All electrical connect ons between the gondola and the recording and control rooms were accomplished via shielded sl p rings

34 C) passing through the glass reference electrode part of the assembly was recorded continuously for correction of the arterial pH to 37 C using a factor of 0 0147 per degree C (ROSENTHAL 1948) 1/15 M phosphate buffers for calibration of the pH unit were prepared according to HASTINGS and SENDROY (1924) and VAN SLYKE WEISIGER and VAN SLYKE (1949) The drift of the pH recording was negligible over a period of several hours For convenient calibration the phosphate buff r solutions were kept at constant temperature in the centrifuge gondola near the pH-oximeter sensing assembly as shown in Fig 3 (C) For measurement of arterial O_2 saturation a cuvette oximeter (WOOD GERACI and GROOM 1948) was used in combination with a logarithmic amplifier (WIEDERHIELM 1956) and yielded a linear response so that only two points were needed for calibration Over the recording periods used in the experiments the drift of the oximeter recording was virtually nil Arterial blood was drawn continuously from the left radial artery via an indwelling Teflon catheter (BARR 1961) through the pH oximeter sensing assembly which was mounted in the gondola in front of the subject Its position was adjusted to approximately correspond to the level of the subject s diaphragm In this way changes in the arterial pressure head at the inflow of the assembly during acceleration were minimized (*cf* LAWTON 1961)

The rate of blood flow through the assembly was kept constant at 8 ml/min by means of the roller pump The time from the opening of the stopcock on the arterial catheter prior to the centrifuge run until the end of the run never exceeded 15 min Thus the blood loss during these periods was limited to 120 ml

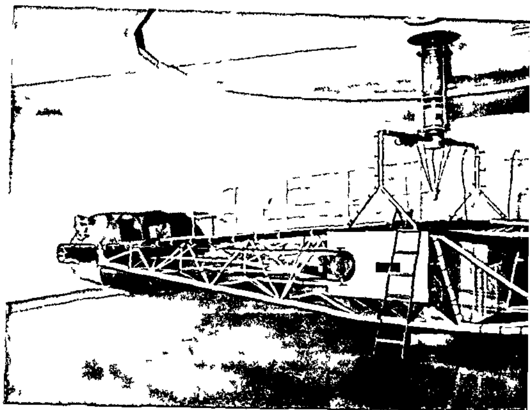


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Arterial pH and O_2 saturation were recorded continuously and simultaneously by means of a pH oximeter assembly The physical dimensions dynamic response and electrical circuitry used with this assembly as well as the methods of calibration have been described elsewhere (BARR and BJURSTEDT 1963) The temperature of the blood (about

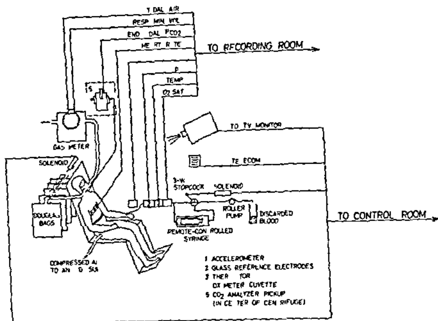


Fig 2 Schemat c representat on of experimental set up in the centrifuge gondola All electrical connections between the gondola and the reco ding and control rooms were accomplished via sh elded sl p rings

34 C) passing through the glass reference electrode part of the assembly was recorded continuously for correction of the arterial pH to 37 C using a factor of 0 0147 per degree C (ROSENTHAL 1948) 1/15 M phosphate buffers for calibration of the pH unit were prepared according to HASTINGS and SENDROY (1924) and VAN SLYKE WEISSIGER and VAN SLYKE (1949) The drift of the pH recording was negligible over a period of several hours For convenient calibration the phosphate buffer solutions were kept at constant temperature in the centrifuge gondola near the pH-oxim ter sensing assembly as shown in Fig 3 (C) For measurement of arterial O_2 saturation a cuvette oximeter (WOOD GERACHI and GROOM 1948) was used in combination with a loga rithmic amplifier (WIEDERHIELM 1956) and yielded a linear response so that only two points were needed for calibration Over the recording periods used in the experi ments the drift of the oximeter recording was virtually nil Arterial blood was drawn continuously from the left radial artery via an indwelling Teflon catheter (BARR 1961) through the pH-oximeter sensing assembly which was mounted in the gondola in front of the subject Its position was adjusted to approximately correspond to the level of the subject s diaphragm In this way changes in the arterial pressure head at the inflow of the assembly during acceleration were minimized (cf LAWTON 1961)

The rate of blood flow through the assembly was kept constant at 8 ml/min by means of the roller pump The time from the opening of the stopcock on the arterial catheter prior to the centrifuge run until the end of the run never exceeded 15 min Thus the blood loss during these periods was limited to 120 ml

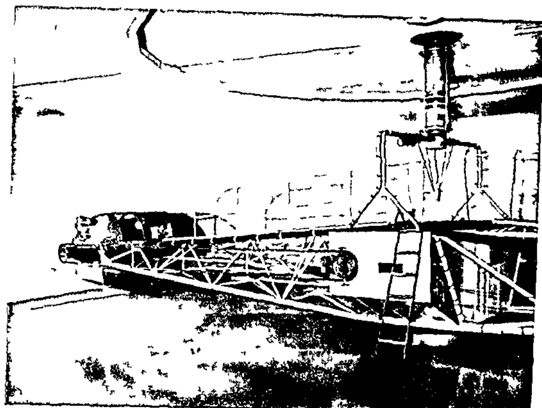


Fig 1 View of gondola and part of the superstructure of the human centrifuge at the Karolinska Institutet

Techniques

Centrifuge

All trials were run in the human centrifuge (Fig 1) at the Karolinska Institutet, the subjects were seated in the gondola that is suspended at one end of the double arm superstructure 7 3 m from the center of rotation On starting the centrifuge the gondola swings out so that when a constant speed has been attained its floor remains perpendicular to the direction of the resultant vector For a description of the centrifuge see GOTZLINGER and HELSING (1955)

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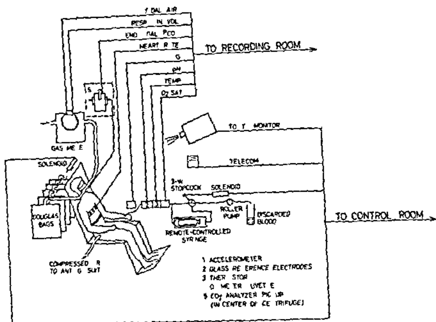


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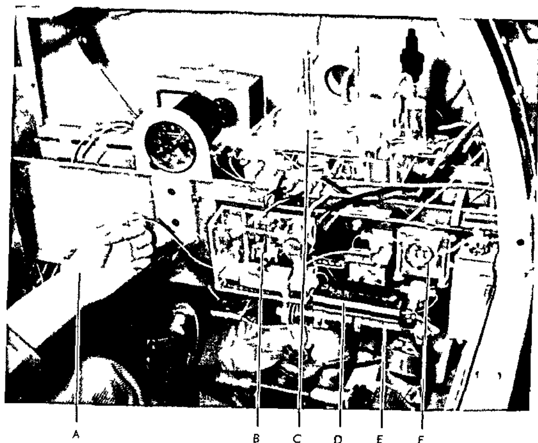


Fig 3 View of the interior of the centrifuge gondola showing part of the instrumentation used for continuous recording of arterial O_2 saturation and pH
A Teflon catheter in radial artery B pH sensing unit with shielded glass electrode between reference electrodes in Lucite housing C Thermostat regulated water bath with phosphate buffer solutions D Oximeter cuvette E Remote controlled syringe for blood sampling F Roller pump

The dynamic responses of the two recording systems were adjusted so as to attain 90 % of full deflection in 8 sec when square wave changes in pH and O_2 saturation were produced *in blood drawn through the radial artery catheter and sensing units*

Inspired gas volumes were recorded quantitatively by connecting the subject (wearing a modified aviator s oxygen mask with a leak proof facepiece) to a modified dry *gas meter (Nordgas Co Stockholm) The gas meter was provided with a photocell* arrangement which transformed the flow of gas into a train of electrical pulses each pulse representing a constant volume (in the present experiments 25 9 ml) of gas *By an electronic two channel pulse adding device described elsewhere* (BJURSTEDT and LONN 1960) (a) each inspiration and (b) the sum of inspired volumes over half minute periods (stair case curve) could thus be recorded At the actual rates of flow *the total resistance through the gas meter tubings and valves did not exceed* 40—45 mm H_2O as measured at the mouth of the subject

The expired gas was collected in a series of small Douglas bags for subsequent analysis For these bags a *special plastic foil* (Cryovac) was used which is less permeable to oxygen carbon dioxide and water vapor than is polyvinylchloride These light

weight bags were positioned behind the back rest of the seat and could be successively connected to the expiratory tube by means of a remotely controlled solenoid-operated 5 way stopcock

End tidal CO_2 tension was automatically obtained by employing a breath by breath gas sampling device (BRISMAR HESSER and MATELL 1962) and a low resistance valve assembly connected to the aviator s mask

The end tidal samples were drawn through a long fine bore stainless steel tube (length 10 m inside diameter 1 8 mm) to the sensing unit of a CO_2 infrared analyzer (Beckman Spinco model LB-1) which was suspended in the center of the centrifuge because its performance was impaired when used in the gondola during G stress The flow of gas was kept constant at 100 ml/min There was a 22 sec delay because of the long tubing before the CO_2 analyzer revealed changes in the end tidal P_{CO} However a step change in the CO_2 tension at the proximal end of the steel tube was 90 % reflected in the recordings within an additional 2 sec The CO_2 meter was calibrated after each experiment by using a series of known mixtures of CO_2 in air saturated with water vapor

Heart rate was recorded from electrodes attached to the chest and connected to an instantaneous cardiotachometer (STURM and WOOD 1947)

The G level was recorded by an accelerometer of the strain gage transducer type mounted at approximately the subject s heart level.

The information gathered by the instruments on the centrifuge was transmitted via a slip-ring system to mirror galvanometers (Gebruder Ruhstrat Göttingen West Germany) in a separate recording room Continuous photokymographic records (45 cm wide) of the galvanometer deflections were taken at a paper speed of 1 25 mm/sec

The subject s face was watched by a closed circuit TV monitor A loud speaker communication system was also used as a safety precaution The subject was instructed not to talk during the experiment except in case of emergency

Laboratory methods

Expired air collected in plastic bags by activation of the solenoid-operated 5-way stopcock was analyzed in duplicate for O_2 and CO_2 according to SCHOLANDER (1947)

O_2 and CO_2 contents of the arterial blood which was sampled by means of a solenoid operated syringe were analyzed in duplicate using the method of VAN SLYKE and NEILL (1924) The maximal difference between duplicates did not exceed 0 2 vol%

Calculations

Expired minute volume ($\dot{V}_E$) was calculated from the observed inspired minute volume with due correction for R and the relative humidity of the inspired gas

Effective alveolar ventilation (Eff $\dot{V}_A$) was obtained from the alveolar ventilation equation substituting arterial for alveolar P_{CO}

Anatomical dead space ($V_{D_{anat}}$) indicates the volume of the conducting airways including the effective external dead space of mask and valve as calculated by the BOHR equation using the end tidal P_{CO} on the understanding that the end tidal gas sample approximates a gas mixture composed of the actual contributions of each alveolus to the expired gas (RILEY and COURNAND 1951)

Table I Individual data

Subject	Age (years)	Height (cm)	Weight (kg)	Blood pressure (*mm Hg*)	Hb conc (g/100 ml blood)
AS	30	179	82	125/85	14.7
AL	26	183	81	135/90	14.5
PL	29	191	88	140/95	15.3
LL	28	184	76	120/90	13.8
NO	36	180	76	140/95	15.0
KJ	26	178	70	125/80	14.0
CB	22	181	79	125/80	14.6
NH	28	173	73	115/85	15.4
KE	23	182	64	145/90	14.0

Physiological dead space ($V_{D_{phy}}$) is used to mean the portion of expired gas which does not remove CO_2 from the pulmonary blood flow. It was calculated using the BOHR formula substituting arterial for alveolar CO_2 (RILEY and COURNAND 1951) and thus includes the effective external dead space.

Alveolar dead space ($V_{D_{alv}}$) is defined as the difference between the physiological and anatomical dead spaces (*cf* SEVERINGHAUS and STUPFEL 1957).

Effective alveolar O_2 tension (Eff $P_{A_{O_2}}$) was obtained from the alveolar gas equation substituting arterial for alveolar CO_2 (RILEY *et al* 1946).

Arterial O_2 tension ($P_{a_{O_2}}$) was calculated from the recorded arterial O_2 saturation and pH_{37} using the line charts of SEVERINGHAUS (1958).

Arterial CO_2 tension ($P_{a_{CO_2}}$) was computed from the $pH_{37°}$ values and the plasma CO_2 content by means of the Henderson Hasselbalch equation, the serum pK'_3 for *carbonic acid being obtained from the nomogram of* SEVERINGHAUS, STUPFEL *and* BRADLEY (1956). The solubility coefficient for CO_2 in plasma was assumed to be 0.521. The plasma CO_2 content was estimated from the observations of CO_2 content, hemoglobin concentration, O_2 saturation and pH of whole blood, using the nomogram of VAN SLYKE and SENDROY (1928) and appropriate correction factors (see PETERS and VAN SLYKE 1931, p. 939). The same procedure was used to estimate $BHCO_{3,t}$ (the plasma bicarbonate content of blood under standard conditions, *i.e.* at 37°, pH 7.40 and saturated with oxygen).

Total venous admixture to the pulmonary veins expressed as percentage of cardiac output was calculated according to BARTELS *et al* (1959, p. 336). For this purpose the $a - \bar{v}$ O_2 difference was assumed to be 5 volume per cent during the pre-run period.

Subjects and Experimental Procedures

The experiments were performed on *9 healthy male subjects. Age and other individual data are given in* Table I. All subjects were volunteers from a wing of the R. S. Air Force where they served as fighter aircraft navigators on active duty. They were in good physical condition. Since they all had several hundred flight hours mostly on high

performance aircraft they were all familiar with the subjective effects of headward acceleration

Each subject came to two sessions with a few days interval between The first session was devoted to acquainting the subject with the experimental conditions an in doctrination experiment was performed in which the subj ct had a trial run on the centrifuge

The second session was devoted to the main experiment which was performed in the morning the subject having previously had a l ght meal After donning his personal anti G suit the subject was provided with chest electrodes for subsequent use with the cardiotachometer He then rested supine on a couch while a Teflon catheter especially designed for use in centrifuge experiments was introduced into the left radial artery A description of the radial artery catheter as v ell as directions for its use have been published elsewhere (BARR and SOILA 1960 BARR 1961) Any subjective inconvenience that would normally occur with an indwelling hypodermic needle was avoided even if the subject inadvertently flexed his wrist The subject was allowed to rest for at least 20—30 min and was given 150 mg heparin intravenously to prevent clotting in the pH-oximeter sensing assembly during the ensuing experiment

The subject then walked to the centrifuge room where he was tied to the seat of the gondola the backrest of the seat was inclined backwards 15 from the vertical and the subject s occiput was in contact with a head rest so that the head and neck were afforded minimal support in an approximately vertical position The anti G suit was connected to the automatic G valve except in experiments where the effects of absence of counter pressure were studied The modified aviator s mask was then donned and tested until free from leakage All electrical connections with recording instruments were checked and the radial artery was finally connected to the pH-oximeter assembly For this purpose the arm was supported with the elbow slightly flexed so that the total length of the tubing needed between artery and sensing units was 25 cm (*cf* Fig 3)

Once the arrangements and connections in the gondola had been completed the subjects were at ease

Standard Experiments Design and Procedure

Excepting certain additional experiments which are described under Methods (p 12—13) and in Comment (p 38—39) the main results of the present investigation (p 21—28) were obtained from experiments which will subsequently be referred to as standard experiments These experiments were designed for one single centrifuge run at + 5 G for 2 min Subjects were breathing air throughout the experiment except during the post run calibrations of the pH-oximeter recording system and wore an anti G suit (similar to the USAF Type G 4A) which was automatically inflated to about 270 mm Hg during the period of acceleration The design and procedure of the standard experiments were as follows

A Pre Run Period Upon completion of preparations described above the subject first rested for at least 10 min in the gondola during which period the continuous photokymographic recording of arterial O_2 saturation pH ventilation end tidal P_{CO} and heart rate was begun By activation of the solenoid operated devices expired gas was then collected in the first Douglas bag for a period of 2 min arterial blood being simultaneously sampled for 1 min The period during which the first arterial blood sample was taken will be referred to as the pre run period

B Run Period After an additional 5 min rest, during which the bag and syringe were removed for analysis and another syringe placed in position the centrifuge was started The plateau level of acceleration of + 5 G was attained after 10—12 sec and was maintained for 2 min After 45—50 sec at + 5 G, expired air was collected in the second bag during the next minute, arterial blood was let into the syringe for a corresponding period of time This one minute period at the end of the run will be referred to as the run period

C Post Run Period When the centrifuge had again come to a stand still the subject remained seated in the gondola for the next phase of the experiment The second bag and syringe were removed for analysis and a third syringe connected After the subject had rested for about 8 min, expired air was collected in the third bag during a period of 2 min arterial blood being simultaneously sampled for 1 min The period during which the third arterial sample was taken will be referred to as the "post run period

Note that all values given in Tables II and III, and in Figs 6 7 and 8 refer to time averages over the pre run, run and post run periods as defined above

Calibrations of pH Oximeter Recording Systems

In order to avoid unnecessary blood loss before and during the standard experiments the oximeter was calibrated at the end of each experiment For this purpose the subject inhaled 10 % O_2 in N_2 until the fall in the oximeter tracing had begun to level off arterial blood was sampled and subsequently analyzed in order to obtain the low HbO_2 point on the calibration curve After a maximum of 7—8 min on low O_2 the subject was shifted to 100 % O_2 and instructed to make several deep inspirations to prevent any residual desaturation due to persisting collapse of alveoli

Upon completion of the oximeter calibration the subject was disconnected from the instruments The pH sensing unit was calibrated against the buffer solutions kept at constant temperature in the gondola

VI RESULTS

The major part of the results are described below under two headings (1) Periods of Relative Stability and (2) Transitional Effects The primary information was obtained from continuous photokymographic records and from analyses of expired air and arterial samples collected as described under Standard Experiments Design and Procedure (see p 19) A segment of a typical record obtained during the standard experiment (a single exposure to +5 G for 2 min) is shown in Fig 4

Periods of Relative Stability

For the various parameters studied group means of individual time averages over one minute periods were computed for (A) pre run (about 5 min prior to acceleration) (B) run (last minute of acceleration) and (C) post run periods (at the end of 8—10 min post acceleration) These one minute periods were assumed to represent periods of relative stability under the control condition during acceleration and during a phase suitable for studying possi ble after-effects respectively The data obtained for the three periods are summarized in Table II

Pulmonary ventilation The expired minute volume V_E increased markedly in all subjects during the period of acceleration The group mean increased from 8 6 to 20 8 l BTPS (increase = 142 per cent) In the post acceleration period the mean V_E had again decreased to slightly below the pre acceleration control level this difference not being statistically significant

Tidal volume and respiratory rate Both parameters were found to increase with acceleration The mean tidal volume V_T reached 1 033 ml from a control value of 639 ml (the increment was significant $p < 01$) The respira tory rate f rose by 55 per cent the mean rate increasing from 13 5 to 20 9 breaths per min during the run period

In the post run period V_T was somewhat below its pre run value whereas f was essentially the same as during the pre run period

The alveolar ventilation (V_A) showed a somewhat greater increase during acceleration (mean increment = 160 per cent) than V_E By contrast the effective alveolar ventilation Eff V_A obtained by substituting arterial for alveolar P_{CO_2} in the alveolar ventilation equation did not increase by more than 95 per cent

In the post run period both V_A and Eff V_A fell to values significantly below the pre run averages

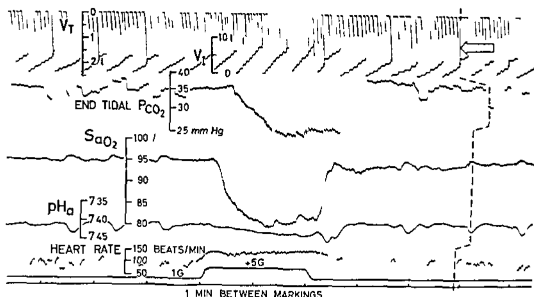


Fig 4 Segment of photokymographic record (scale = 1/6 of original) typifying the primary information from which main results of the investigation were deduced Note the marked lowering of the arterial O_2 saturation during the standardized exposure to + 5 G for 2 min with anti G suit in spite of concomitant increase in ventilation Note also the slight alkalotic shift in the arterial pH indicating that the shunt effect with regard to CO_2 is masked by state of relative hyperventilation Dashed line indicates lags after which pulmonary events (exemplified by deep inspiration at arrow) are reflected in the tracings

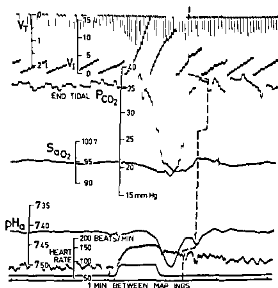


Fig 5 Photokymographic recording of the responses of ventilatory parameters arterial O_2 saturation arterial pH and heart rate during exposure of healthy man to acceleration of + 5 G for about 45 sec without inflation of the anti G suit Note that the O_2 saturation falls only slightly Ventilation increases greatly and is associated with a marked fall in end tidal CO_2 tension The run was terminated because of blackout and impending loss of consciousness

Arterial O_2 saturation and O_2 tension Recorded group means for S_{aO_2} and for calculated P_{aO_2} decreased during the run period by 8 8 per cent to 87 4 per cent (lowest recorded individual one minute mean value 81 0 per cent) and by 32 1 mm Hg to 58 0 mm Hg (lowest one minute mean value 41 mm Hg)

In the post run period all individual time averages for arterial S_{O_2} and P_O were below pre run values Group means were 94 8 per cent and 79 4 mm Hg respectively both decrements being significant ($p < 01$)

Effective alveolar O_2 tension The mean Eff P_{A_O} was found to increase from 103 2 to 112 0 mm Hg during acceleration the increment being highly significant ($p < 001$) For the post run period practically the same value was obtained as for the pre run period

Arterial pH The individual records showed relatively small changes during the course of the run The normal mean control value of 7 407 gives objective confirmation of the relaxed state of the subjects A small but statistically significant shift towards alkalosis was observed during the run (mean change = + 0 027 of a pH unit $p < 01$) In the post run period the mean value was 0 014 of a pH unit more acid than in the pre run period ($p < 001$)

End tidal CO_2 tension The breath by breath recordings of end tidal P_{CO} showed a gradual decrement during acceleration The mean pre run control value was 34 5 mm Hg and the mean during acceleration 23 9 mm Hg During the post run period the group mean (32 1 mm Hg) was slightly but significantly lower than in the pre run period ($p < 01$)

Arterial CO_2 tension and $BHCO_{3S}$ During acceleration there was a decrease in the calculated arterial CO_2 tension P_{CO_2} from an average of 38 1 to 35 5 mm Hg ($p < 05$) The standard bicarbonate $BHCO_{3,t}$ remained con stant during acceleration During the post run period P_{CO} returned to the mean pre run value whereas the buffering capacity of the arterial blood was slightly decreased as compared with the pre run and run values

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Notes to Table II (p 24—25)

Calculated as ($V_{CO} \times 863$)/End tidal P_{CO}

Calculated as ($V_{CO} \times 863$)/P_{CO_2}

Computed from the BOHR formula, substituting arterial for alveolar CO (Effective external dead space of 70 ml included)

Calcul ted as ($V_E - V_A$)/f (Effecti e external dead space of 70 ml in luded)

Calculate l as $V_{D_{phys}} - V_{D_{an}}$ t

$BHCO_{3S}$ = plasma b carbonate content of blood under standard condition, i e at 37 C, pH 7 40 and saturated with oxygen.

Computed from the HENDERSON HASSELBALCH equation.

Calculated from arteri l O saturation and pH by use of the line charts of SEVERINGHAUS (1958)

Calculated as $P_{IO} - P_{ACO}\left(F_{IO} + \frac{1 - F_{IO}}{R}\right)$

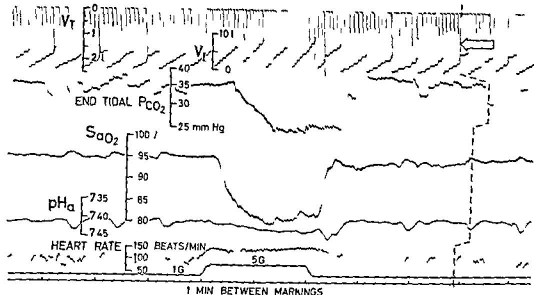


Fig 4 Segment of photokymographic record (scale = 1/6 of original) typifying the primary information from which main results of the investigation were deduced Note the marked lowering of the arterial O_2 saturation during the standardized exposure to + 5 G for 2 min with anti G suit in spite of concomitant increase in ventilation Note also the slight alkalotic shift in the arterial pH indicating that the shunt effect with regard to CO_2 is masked by state of relative hyperventilation Dashed line indicates lags after which pulmonary events (exemplified by deep inspiration at arrow) are reflected in the tracings

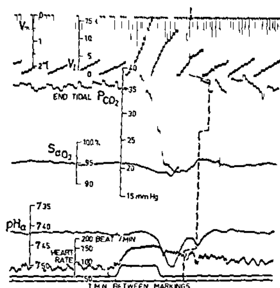


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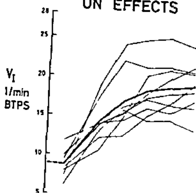
V_{D_1} (ml BTPS)	$\frac{V_{D_{IV}}}{V_T - V_{D_{mt}}} \times 100$	Art pH	$BHCO_3$ (mM/l)	Art O_2 sat ( )	P_{CO_2} (mm Hg)	End tidal P_{CO_2} (mm Hg)	P_{CO_2}—End tidal P_{CO_2} (mm Hg)	P_{O_2} (mm Hg)	Eff $P_{A_{O_2}}$ (mm Hg)	Eff $P_{A_{O_2}}$—P_{O_2} (mm Hg)	Heart rate (beats/min)
9	9	9	9	9	9	9	9	9	9	9	8
37	9	7407	242	962	331	34.5	36	901	103.2	131	78
±6	±1	±005	±5	±1	±7	±8	±6	±7	±13	±14	±3
6	2	7.381	209	958	353	304	8	86.5	97.3	66	67
−68	−14	−7425	−263	−967	−417	−374	−57	−947	−107.2	−196	−89
9	9	9	7	9	9	9	9	9	9	9	8
216	32	7434	245	874	35.5	239	116	580	1120	54.2	132
±14	±2	±009	±6	±19	±11	±6	±10	±53	±19	±44	±8
152	18	7388	216	810	303	214	54	444	99.2	25.8	104
−273	−39	−7467	−267	−970	−431	−27.2	−159	−973	−1181	−681	−172
6	6	7	6	7	6	7	6	7	6	6	8
49	14	7395	230	948	375	321	58	794	1096	243	80
±10	±3	±006	±6	±2	±4	±8	±9	±2.2	±9	±2.2	±5
14	3	7368	207	940	356	284	34	724	99.2	159	63
−85	−24	−7420	−25.2	−955	−38.2	−348	−91	−86.5	−1049	−291	−105
9	9	9	7	9	9	9	9	9	9	9	8
+179	+23	+097	+3	−88	−26	−106	+80	−321	+88	+411	+44
±18	±3	±005	±.2	±1.9	±8	±.8	±10	±51	±16	±47	±7
<001	<001	<01	>05	<01	<05	<001	<001	<001	<001	<001	<001
6	6	7	6	7	6	7	6	7	6	6	8
+10	+6	−014	−.9	−15	+6	−19	+23	−109	−21	+10.5	+2
±3	±2	±007	±3	±3	±4	±4	±.2	±24	±9	±17	±3
<05	<05	<001	<05	<01	>05	<01	<001	<01	>05	<001	>05

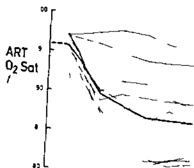
Table II Pre run run (+ 5 G, with anti G suit) and post run periods

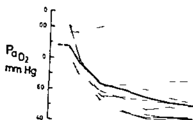
	V_E (l/min BTPS)	V_T (ml BTPS)	f (breaths/min)	V_{O_2} (ml/min STPD)	V_{CO_2} (ml/min STPD)	R	V_A[1] (l/min BTPS)	Eff V_A[2] (l/min BTPS)	$\frac{\text{Eff } V_A}{V_E} \times 100$	$V_{D_{phys}}$[3] (ml BTPS)	$V_{D_{a\,t}}$ (ml BTPS)
Pre run (A)											
n	9	9	9	9	9	9	9	9	9	9	9
$\bar{x} \pm \varepsilon\bar{x}$	8 55 ± 49	639 ± 40	13 5 ± 6	269 ±15	216 ±13	80 ± 02	5 40 ± 32	4 90 ± 31	57 ±2	270 ±19	233 ±18
Range	6 32 −10 56	527 −841	11 6 −16 6	224 −342	158 −277	71 − 91	4 27 −6 71	3 78 −6 49	48 −66	182 −360	145 −310
Run (B)											
n	9	9	9	9	9	9	9	9	9	9	9
$\bar{x} \pm \varepsilon\bar{x}$	20 81 ±1 24	1 033 ±99	20 9 ±1 3	410 ±39	391 ±34	96 ± 03	14 03 ±1 02	9 59 ± 94	45 ±3	543 ±29	398 ±14
Range	15 66 −29 00	719 −1 688	14 1 −26 5	254 −572	273 −553	83 −1 09	9 98 −19 17	6 92 −15 33	38 −64	400 −656	249 −385
Post run (C)											
n	7	7	7	7	7	7	7	6	6	6	7
$\bar{x} \pm \varepsilon\bar{x}$	8 30 ± 52	559 ±39	15 1 ±1 1	252 ±20	195 ±14	78 ± 02	5 23 ± 34	4 63 ± 33	54 ±4	254 ±18	202 ±15
Range	6 25 −9 61	447 −723	12 8 −21 5	196 −337	148 −249	74 − 86	3 89 −6 29	3 41 −5 63	35 −61	205 −315	150 −256
Difference (B−A)											
n	9	9	9	9	9	9	9	9	9	9	9
$\bar{x} \pm \varepsilon\bar{x}$	+12 26 ±1 17	+394 ±88	+7 4 ± 8	+141 ±34	+ 175 ±29	+ 16 ± 03	+8 63 ± 95	+4 69 ± 84	−12 ±2	+ 273 ±30	+95 ±19
p	< 001	< 01	< 001	< 01	< 001	< 001	< 001	< 001	< 001	< 001	< 01
Difference (C−A)											
n	7	7	7	7	7	7	7	6	6	6	7
$\bar{x} \pm \varepsilon\bar{x}$	− 12 ± 37	−71 ± 23	+1 6 ± 8	−14 ±6	−21 ±5	− 03 ± 02	− 26 ± 11	− 54 ± 11	−6 ±1	−4 ±15	−15 ±11
p	> 05	< 05	> 05	< 05	< 01	> 05	< 05	< 01	< 01	> 05	> 05

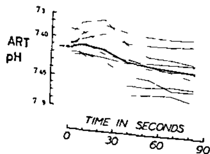
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ON EFFECTS

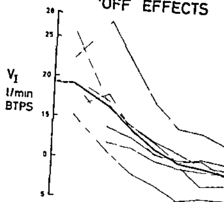


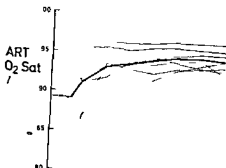


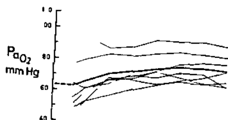


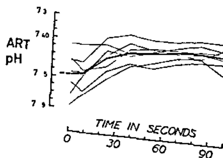


'OFF EFFECTS









O_2 uptake, CO_2 elimination and respiratory exchange ratio Acceleration provoked an increase in the oxygen uptake, V_{O_2}, averaging 52 per cent, and a concomitant increase of 81 per cent in the CO_2 output, V_{CO_2} These values indicate an elevation in the respiratory exchange ratio, R, *from 0 80 to 0 96* During the post acceleration period R returned to the pre run level During this period V_{O_2} and V_{CO_2} decreased to values slightly below their pre run values

Transitional Effects

In order to study more closely the rates of change in the pulmonary ventilation and in the arterial O_2 saturation, O_2 tension and pH occurring after the rise ('on' effects) and fall ('off' effects) of the G force in standard exposures to + 5 G for 2 min, the following analysis was made

Beginning at the moments of starting and stopping the centrifuge, the individual time averages over consecutive 15 sec periods were computed for each parameter The values for V_I arterial O_2 saturation and pH were obtained from the continuous photokymographic tracings, whereas those for the arterial O_2 tension were calculated from O_2 saturation and $pH_{37°}$, using the line charts of SEVERINGHAUS (1958) Before plotting these values against time, *the values for ventilation were smoothed by determining for every* 15 sec, the mean of three consecutive values Furthermore, the values obtained for blood chemical factors were moved along the time axis to allow for the time taken for events in the pulmonary capillaries to be reflected in the O_2 and pH recordings

Fig 6 illustrates the results of these operations, *individual values being* connected by thin lines, and mean values by heavy lines

Inspired minute volume (V_I) started to increase almost immediately after onset of acceleration, 90 per cent of the final mean change being attained within less than one minute It was noted that two of the subjects showed a much more marked increase in ventilation than did the rest of the group Inspection

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Fig 6 On and off effects in (from above) pulmonary ventilation and arterial O_2 saturation O_2 tension and pH following the fast rise and fall of the G force in standard exposures to + 5 G for 2 min with anti G suit (time for attaining the + 5 G plateau as well as for the return was about 12 sec) On effects 9 subjects off effects 7 subjects
Heavy lines show means of individual time averages (*thin lines*) which were determined over successive 15 sec periods
Dashed lines represent group means immediately prior to starting and stopping the centrifuge
Note that all blood chemical changes have been processed from continuous recordings of arterial O_2 saturation and pH after subtracting the time taken for passage of blood from the lungs to the pH oximeter assembly

VII DISCUSSION

Effects on Respiration Arterial O_2 Saturation and pH

It is well known that changes in gravitational vectors normally produce alterations in the respiratory activity in man so that e g a change from the supine to the up right position produces an increase in the respiratory minute volume (for reviews see BROGDON FRANSEEN and HELLEBRANDT 1943 BJURSTEDT et al 1962) A marked increase in respiratory activity has also been observed in man during exposures to headward acceleration (GAUER 1938 LOMBARD ROOTH and DRURY 1948, BROWNE 1958 ROSSANIGO and MEIVERI 1961 BARR 1962)

In the present experiments the mean expired minute volume increased from 8 6 to 20 8 l/min during acceleration (cf Table II) The mean values for expired minute volume during the pre run run and post run periods are graphically depicted in Fig 7 which also shows the concomitant alterations in the effective alveolar ventilation (for discussion of the relations between the two quantities see below p 36)

The hyperpnea was associated with a marked decrease in the O_2 saturation level from a mean of 96 2 to 87 4 per cent (Table II) Such acceleration induced unsaturation has been briefly reported in earlier investigations (p 9—10) The finding of an increased respiratory minute volume in combination with arterial unsaturation indicates an increase in the alveolar arterial O_2 difference the significance of which will be discussed below in a separate section (p 32– 36)

The observation that the marked increase in the respiratory minute volume and the concomitant arterial unsaturation were associated with only a small increase in the mean arterial pH (from 7 407 to 7 434) complicates the analysis of the mechanism underlying the respiratory stimulation Although it is well known that assuming the up right position under normal gravity calls forth an increase in the pulmonary ventilation the cause of postural hyperventilation is not fully understood The concomitant lowering of the alveolar CO_2 tension is in part due to an increased arterial to end tidal CO_2 difference (BJURSTEDT et al 1962) the postural decrease in alveolar CO_2 tension thus being only in part due to the lowering in arterial CO_2 tension that normally accompanies increased ventilation

The strong respiratory stimulation seen during headward acceleration may be due to a number of influences Thus the fall in arterial blood pressure that occurs in the upper part of the body when exposed to gravitational stress (cf WOOD et al 1946) may stimulate respiration by direct influence on the medullary centers by changes in the local blood flow (for review see SCHMIDT

of the original recordings also showed a more marked alkalotic trend in the two cases

The ventilatory 'off' effect was less rapid, especially during the first half-minute The mean curve returned to the pre run level within 90 sec

Arterial O_2 saturation The mean level decreased immediately upon onset of acceleration and reached 90 per cent of the maximal fall within 60 sec During the last 30 sec of the 2 min exposure (not shown in Fig 6) the mean O_2 saturation curve again rose by a few per cent Two of the subjects showed only a small decrease in O_2 saturation during the run

On stopping the centrifuge the return of the mean arterial O_2 saturation was relatively rapid in the beginning, but after about 30—45 sec became gradually slower The return to pre run level was not completed before 90 sec had elapsed

Arterial O_2 tension The alterations in the O_2 tension followed a pattern similar to that of the O_2 saturation Because of the slight change towards alkalosis, the mean O_2 tension fell more than what would otherwise be expected from the decrement in O saturation

The return to normal level after acceleration was relatively slower for mean O_2 tension than for saturation, especially in the beginning and was not completed within the time period studied

Arterial pH Fig 6 demonstrates some advantages of using continuous recording techniques for arterial pH As can be seen the subjects generally showed greater inter individual than intra individual differences during the periods studied It is evident that the individual pH levels remained remarkably stable in spite of the great changes in ventilation Since pH is the negative logarithm of [H+] the calculation of mean pH values by standard methods would ordinarily not appear correct However since in this case the differences between the values did not exceed 0 1 of a pH unit the error introduced was considered negligible The general trend as described by the mean curve, was towards a slight acidotic shift during the first 30 sec of acceleration and a subsequent more persistent alkalotic change during the remainder of the run

The mean pH off effect was a relatively rapid return to the pre run level with a slight but somewhat protracted overshoot

In summing up the transient changes described and illustrated in the present section, it can be stated that with the standard exposure used in the present experiments (+ 5 G for 2 min), most of the changes seen in $\dot{V}_I$, and in arterial O_2 saturation and O_2 tension were completed within 60 sec, whereas the return to pre run levels after stopping the centrifuge was not complete at the end of the time period studied except for arterial pH

The changes in alveolar arterial gas tension differences that were observed in the present experiments are graphically depicted in Fig 8 The arterial to end tidal CO_2 difference increased by an average of 8 0 mm Hg (*cf* Table II) Assuming the transmembrane CO_2 gradient to be negligible the observed rise may be ascribed to at least three factors (1) underperfusion or overventilation of alveoli (2) underventilation or overperfusion of alveoli and (3) extra alveolar shunts Theoretically all three factors may have contributed to establishing the increment in the CO_2 difference There is evidence that normal gravity produces regional (apical) underperfusion rather than overventilation (MATTSON and CARLENS 1955 MARTIN and YOUNG 1957 RILEY *et al* 1959 WEST and DOLLERY 1960)

The mechanism underlying the present results is however to some extent obscured by the fact that information on the changes in cardiac output during acceleration is as yet limited Considering that the arterial to end tidal CO_2 difference is also affected by changes in total flow to the lungs (RILEY *et al* 1959) the extent to which a decrease in cardiac output might have contributed to the CO_2 difference thus remains open to question The relative importance of changes in cardiac output is clearly illustrated by the experiments of GERST RATTENBORG and HOLADAY (1959) who observed a substantial increase in the alveolar dead space in supine anesthetized dogs after the total blood flow had been diminished by hemorrhage

Since both a regional decrease in the pulmonary blood flow and a diffuse closing of capillaries throughout the lungs are capable of producing a CO_2 difference and both these effects may theoretically be produced by gravitational stress one may conclude that much larger CO_2 differences would have been obtained had the present experiments been performed at high G levels but without the use of anti G suits This is borne out by the observation of HOWARD (1959) that cardiac output decreased by about 40 per cent in a subject exposed to headward acceleration of 2 4 G with the legs extended Unfortunately cardiac output measurements at higher levels of acceleration and with the use of anti G suits have indicated wide variations Thus LINDBERG *et al* (1960) with the dye dilution technique obtained a mean decrease of 22 (range 4 to 45) per cent after 20—40 seconds of headward acceleration at 4 G Inflating a G 3A suit increased the range of alterations in cardiac output but did not change the average figures significantly

The use of the anti G suit in the present experiments presumably prevented a precipitous fall in cardiac output during the runs However it is likely that cardiac output was reduced because of the rather inefficient protection afforded by this device It can therefore not be excluded that a diffuse closing of capillaries throughout the lungs secondary to decreased cardiac output contributed to the observed arterial to end tidal CO_2 difference

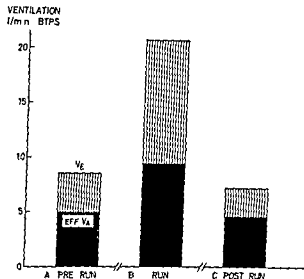


Fig 7 Expired minute volumes ($\dot{V}_E$, total height of columns) and effective alveolar ventilation (Eff $\dot{V}_A$ black part of columns) before during and after exposure to +5 G during 2 min (mean values of the ensemble of time averages over one minute periods) For statistical data see Table II

1956) Also, hypotension at the level of the carotid sinus region may give rise to respiratory reflexes of chemoceptive or baroceptive origin (*cf* EULER and LILJESTRAND 1937, HEYMANS and NEIL 1958) Such stimulation may well become more intense through ischemic excitation of carotid chemoreceptors secondary to reduced blood flow to these structures (*cf* LANDGREN and NEIL 1951) Evidence that carotid chemoreflexes may be responsible for most of the hyperventilation seen in anesthetized dogs was obtained by BARR, BJURSTEDT and COLERIDGE (1959a), who found that respiration decreased during headward acceleration after denervation of the carotid chemoreceptors

In general, the arterial pH remained remarkably constant during acceleration (*cf* Table II) As will be discussed in a subsequent section, a considerable deterioration occurred in the efficiency of the over all pulmonary gas exchange, which tended to counteract the respiratory alkalosis that would otherwise be expected from the great increase in total pulmonary ventilation It is likely that such reduction of the arterial alkalosis contributed to increasing any respiratory stimulation caused by concomitant arterial hypoxemia (*cf* NIELSEN and SMITH 1951)

Arterial to End Tidal CO Difference and Dead Spaces

Variations of V/Q ratio among alveoli should result in an alveolar arterial CO_2 difference (RILEY and COURNAND 1951 SEVERINGHAUS and STUPFEL 1957) That such variations occur as a result of the action of normal gravity was first made likely by the use of differential spirometry (*cf* p 8—9) The occurrence of a significant CO_2 difference in the standing position has only recently been demonstrated independently by ULMER and REICHEL (1961) and by BJURSTEDT *et al* (1962)

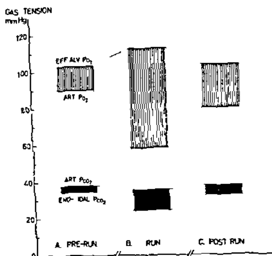


Fig 8 Alveolar arterial O and CO differences before during and after e posu e to + 5 G for ? m n (mean alues of the en semble of t me a rag s over one min ıte per od)

Height of st ped areas equals Eff PAo —P o

Height of black easequals P co — e d tidal P_{CO_2}

Fo stat t cal data see Table II

end tidal to arterial O_2 difference was found to increase from a mean value of 18 1 to 67 0 mm Hg

This over all O_2 difference can basically be referred to the same three factors (*cf* p 31) which were responsible for the CO_2 difference (FARHI and RAHN 1955 BARTELS *et al* 1959 HAAB PIIPER and RAHN 1960) provided the transmembrane gradient for O_2 is negligible during air breathing (*cf* RILEY and COURNAND 1951) During centrifugation the CO_2 difference was mainly the result of impaired apical perfusion of the lungs with the development of a considerable alveolar dead space However the increment in the alveolar dead space contributed only to a minor part to the increase in the over all O_2 difference (about 10 mm Hg) The greater part of the overall O_2 difference was due to the effective alveolar to arterial O_2 difference (averaging 54 2 mm Hg Table II) which represents that portion of the O_2 difference which is caused by the addition of venous blood to the arterialized blood

The complex picture of pulmonary gas exchange during acceleration may be visualized schematically by assuming that headward acceleration results in regional V/Q ratios of the following main types and locations (1) an upper region which is ventilated but not perfused (2) a middle region ideally ventilated and perfused and (3) a dependent region which is perfused but not ventilated

The large increase in the effective alveolar to arterial O_2 difference graphically depicted in Fig 8 does not necessarily imply a corresponding increase in the total venous admixture since the desaturation of the arterial blood is the result of both the volume of the venous blood admixed and its oxygen content To estimate the relative importance of the latter

Accordingly, there are reasons to believe that the substantial increase in the CO_2 difference during G stress in the present experiments was not only due to downward displacement of blood in the pulmonary circulation but also to similar events in the systemic circulation, thereby reducing the effective volume of blood available for the circulation It may be noted in passing that gravitational displacement of blood volume within the systemic circulation can readily be avoided if the body is submerged in water while exposed to G stress Since cardiac output would then be wholly unaffected, any increase in the arterial to end tidal CO_2 difference must be referred solely to regional displacement of blood within the lungs Immersion experiments would thus be ideal for studies of gravitational rearrangement of pulmonary V/Q ratios

Exposure to G forces acting perpendicular to the long axis of the body (*e g* in the 'astronaut position') would offer a situation similar to that of headward acceleration with the body submerged in water In the first mentioned condition neither cardiac output nor over all alveolar ventilation are significantly changed (*cf* Hershgold and Steiner 1960, Steiner, Mueller and Taylor 1960, Wood *et al* 1961, Zechman, Cherniack and Hyde 1960) To date, no information is available on the CO_2 difference under transverse acceleration Theoretically, any discrepancy between end tidal and arterial CO tension in this situation would reflect purely regional alterations of V/Q ratios within the lungs

The physiological dead space, as calculated from the Bohr formula, increased during acceleration by 273 ml on an average whereas the alveolar dead space was enlarged by 179 ml (*cf* Table II) This portion of the dead space, *i e* the alveolar volume that did not exchange with blood, increased to nearly six times its pre run value The increase in the alveolar dead space may be ascribed to alterations in the ventilation perfusion relationships both throughout the lungs and regionally If all alveoli are arbitrarily divided in two groups, one with ideal exchange with the blood, the other unperfused, and if the distribution of the ventilation is assumed to remain unchanged (*cf* Severinghaus and Stupfel 1957), it is possible to calculate that the increment in the gravitational vector blocked the perfusion to roughly one third of the total number of alveoli (*cf* Table II $V_{D\,al}/V_T - V_{D\,an}$)

Alveolar to Arterial O_2 Difference and Venous Admixture

The lowering of the oxygen saturation in the mixed arterial blood observed in this investigation under G stress was not the result of an alveolar hypoventilation In fact a relative alveolar hyperventilation was present as judged by the slight alkalotic trend in the arterial pH This implies that the arterial hypoxemia was associated with a considerable alveolar to arterial O_2 difference Assuming end tidal gas to have the same R as mixed expired gas the

In practice available data do not permit any conclusions as to the magnitude of this decrease in cardiac output Therefore the total venous admixture has been calculated at three different levels of cardiac output during acceleration (Table III) It is believed that the most reasonable values are those given in column (b) assuming a 20 per cent reduction of $\dot{Q}$ during acceleration The total venous admixture in the run period would then amount to about 1/5 of the total blood flow and in consequence cause a considerable O_2 deficit in the mixed arterial blood The concomitant arterial O_2 deficit thus amounted to about 25 per cent of the measured oxygen uptake (Table III)

The total venous admixture as calculated in the present investigation includes the blood flow through extra alveolar (anatomical) shunts as well as the flow from underventilated or overperfused alveoli Since there are no obvious reasons to assume an increased blood flow through existing anatomical shunts in healthy individuals during acceleration it is felt that the major part of the increment in venous admixture is due to alveolar shunts Such shunts seem likely to develop in the dependent parts of the lung as a result of the increased effective weight of the blood Direct evidence for such displacement of blood has been presented in X ray studies (p 10)

The use of the anti G suit during acceleration would tend to counteract excessive pooling of blood in dependent and distensible portions of the systemic circulation It can thus be anticipated that the prevention of such pooling *e g* by submerging the body in water or by applying the G force perpendicular to the long axis of the body would result in considerably larger shunts than in the extreme case of subjecting the body to headward acceleration without anti G suit and with the legs extended By the same token it may be assumed that the use of anti G suits in the present experiments exaggerated the shunt effect that would otherwise have developed as the consequence of increasing the effective force of normal gravity During exposure to *transverse* G where no reduction of the blood flow occurs a 50 per cent "shunt effect was demonstrated at 8 G (STEINER and MUELLER 1961)

The above considerations regarding the establishment of a large O_2 difference during acceleration rest on the assumption of a negligible alveolar trans membrane O_2 gradient The validity of this assumption can however be questioned In the lowermost regions of the lung the intracapillary hydrostatic pressure may be sufficiently increased to cause a local *edema* resulting in impaired gas diffusion The methods used in the present investigation do not permit differentiation between regional rearrangements *of* $\dot{V}/\dot{Q}$ *ratios and* regional diffusion impairment in the production of alveolar arterial O_2 differences

Congestion in the lowermost subdivisions of the lung during headward acceleration especially if combined with regional pulmonary edema would likely cause atelectasis *in these regions* X ray studies suggest such changes following headward acceleration (ERNSTING 1960) The reported subjective symptoms

Table III Calculated total venous admixture and arterial O_2 deficit resulting from exposure to + 5 G for 2 min Columns (a), (b) and (c) show values at an assumed cardiac output (Q) of 100 80 and 60 % of ' Pre run Q [1], respectively

	Total venous admixture[2] (%)			Arterial oxygen deficit[3] (ml O_2/min STPD)		
	(a)	(b)	(c)	(a)	(b)	(c)
Pre run (A)						
n		9			9	
$\bar{x} \pm \varepsilon\bar{x}$		5 8± 9			17± 2	
Range		3 7–12 1			9–33	
Run (B)						
n	9	9	9	9	9	9
$\bar{x} \pm \varepsilon\bar{x}$	23 4± 3 6	19 8± 3 1	15 9± 2 6	130± 27	103± 21	78± 16
Range	3 7–41 8	3 1–36 3	2 3–30 2	22–269	17–213	13–16[illegible]
Post run (C)						
n	6	6	–	6	6	–
$\bar{x} \pm \varepsilon\bar{x}$	11 3± 1 3	8 9± 8	–	33± 5	26± 4	–
Range	7 4–16 8	6 0–11 4	–	16– 54	13– 43	–
Difference (B–A)						
n	9	9	9	9	9	9
$\bar{x} \pm \varepsilon\bar{x}$	17 6± 3 7	14 0± 3 2	10 1± 2 7	113 ± 26	86± 21	61± 16
p	< 01	< 01	< 01	< 001	< 001	< 001
Difference (C– A)						
n	6	6	–	6	6	–
$\bar{x} \pm \varepsilon\bar{x}$	6 5± 1 1	4 0± 7	–	18 ± 3	12± 3	–
p	< 01	< 01	–	< 001	< 001	–

[1] Pre run Q calculated as $\frac{\dot{V}_{O_2}}{a-\bar{v}\ O_2\ \text{diff}}$ assuming the a – $\bar{v}$ O_2 difference to be 50 ml l.

[2] Calculated as $100 \times \frac{C - C}{C_c - C_v}$

Calculated as Q $(C_c - C\,)$

components in the establishment of the O_2 difference, it is necessary to assume values either for the cardiac output or for the composition of the mixed venous blood In view of the considerations put forward above in connection with the discussion of the CO_2 difference it is likely that a decrease occurred in the cardiac output during acceleration

In practice available data do not permit any conclusions as to the magnitude of this decrease in cardiac output Therefore the total venous admixture has been calculated at three different levels of cardiac output during acceleration (Table III) It is believed that the most reasonable values are those given in column (b) assuming a 20 per cent reduction of $\dot{Q}$ during acceleration The total venous admixture in the run period would then amount to about 1/5 of the total blood flow and in consequence cause a considerable O_2 deficit in the mixed arterial blood The concomitant arterial O_2 deficit thus amounted to about 25 per cent of the measured oxygen uptake (Table III)

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The use of the anti G suit during acceleration would tend to counteract excessive pooling of blood in dependent and distensible portions of the systemic circulation It can thus be anticipated that the prevention of such pooling *e g* by submerging the body in water or by applying the G force perpendicular to the long axis of the body would result in considerably larger shunts than in the extreme case of subjecting the body to headward acceleration without anti G suit and with the legs extended By the same token it may be assumed that the use of anti G suits in the present experiments exaggerated the shunt effect that would otherwise have developed as the consequence of increasing the effective force of normal gravity During exposure to transverse G where no reduction of the blood flow occurs a 50 per cent *shunt effect* was demonstrated at 8 G (STEINER and MUELLER 1961)

The above considerations regarding the establishment of a large O_2 difference during acceleration rest on the assumption of a negligible alveolar trans membrane O_2 gradient The validity of this assumption can *however* be questioned In the lowermost regions of the lung the intracapillary hydrostatic pressure may be sufficiently increased to cause a local edema resulting in impaired gas diffusion The methods used in the present investigation do not permit differentiation between regional rearrangements of $\dot{V}/\dot{Q}$ ratios and regional diffusion impairment in the production of alveolar arterial O_2 differences

Congestion in the lowermost subdivisions of the lung during headward acceleration, especially if combined with regional pulmonary edema would likely cause atelectasis in these regions X ray studies suggest such changes following headward acceleration (ERNSTING 1960) The reported subjective symptoms

such as coughing and substernal pain experienced by aviators, have also been ascribed to the development of atelectasis or edema during acceleration (LANGDON and REYNOLDS 1961) In the present investigation the persistence of a relatively large effective alveolar to arterial O difference in the post run period (Fig 8) may be taken as an indication of long lasting disturbances in the pulmonary circulation, possibly in part due to residual atelectasis

Effects of Venous Admixture on Arterial CO Tension Consequences in the Calculation of Venous Admixture Effective Alveolar Ventilation and Dead Spaces

In the calculations of the Eff $P_{A_{O_2}}$ and venous admixture, the pulmonary end capillary P_{CO_2} was assumed to be identical with the measured arterial P_{CO_2} This approximation is permissible when the venous admixture is lower than 20 per cent of Q (*cf* BARTELS *et al* 1959) It was found, however, that the venous admixture was of this order of magnitude during acceleration (*cf* Run ' in Table III) It was therefore considered important to evaluate (a) the influence of venous admixture on the arterial P_{CO_2} and (b) the influence of using a corrected end capillary P_{CO} in the calculations of Eff V_A dead spaces Eff $P_{A_{O_2}}$ and venous admixture

In the run period the venous admixture as calculated in Table III, was found to raise $P_{a_{CO_2}}$ by 2 2 mm Hg (group average), irrespective of the assumed value for Q Accordingly, the run period value in Table II for Eff V_A was somewhat underestimated and those for physiological and alveolar dead spaces somewhat overestimated Using a corrected end capillary P_{CO_2} (measured P_{CO_2} reduced by 2 2 mm Hg) the Eff $P_{A_{O_2}}$ was calculated to be 114 mm Hg instead of 112 mm Hg (group means) The values for venous admixture remained, on the other hand essentially unaffected by these corrections

Efficiency of Over All Pulmonary Gas Exchange

During acceleration both the expired minute volume V_E, and the alveolar ventilation, V_A increased (average + 12 3 and + 8 6 l/min BTPS respectively) The effective alveolar ventilation, Eff V_A calculated from the arterial CO_2 tension, also increased but relatively less than V_E the average increment being only 4 7 l/min This is apparent from Table II which also shows that the mean value for the Eff V_A/V ratio decreased from 57 to 45 per cent The relations between the mean values of effective alveolar ventilation and total pulmonary ventilation in the pre run run and post run periods are graphically depicted in Fig 7

The total O_2 uptake increased during the runs from an average of 269 to 410 ml/min, whereas the value was 252 ml/min in the post run period (8—10 min after the exposure) Since it was surmised that an O_2 debt might have occurred during acceleration, additional data were gathered on the behavior

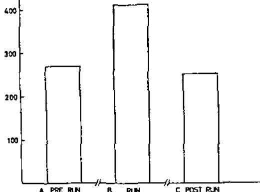


Fig 9 Oxygen uptake per min ($\dot{V}_{O_2}$) before during and after exposure to + 5 G for 2 min (mean values of the ensemble of tim averages over two- one and two-minute periods respectively) For statistical data see Table II

of the O_2 uptake immediately following the exposure The data obtained showed for the group a statistically significant rise of the O_2 uptake during the first minute after acceleration as compared with the group mean during acceleration The decrease in $BHCO_{3_{st}}$ in the post run period indicating the production of acid metabolites during the period of G stress is further evidence for the development of an O_2 debt (Table II) Thus it is evident that the increase in O_2 uptake graphically depicted in Fig 9 was insufficient to prevent the development of an O_2 debt

This O_2 debt may be caused by (1) an increase in the metabolic need for O_2 from the extra work associated with respiration and maintenance of posture under acceleration and (2) insufficient supply of O_2 to the tissues secondary to local circulatory disturbances in the dependent regions of the body and/or the presence of arterial hypoxemia Since STEINER MUELLER and CHERNIACK (1961) have shown that extra work load during exposures to high gravitational stress is associated with an additional uptake of O_2 it is not likely that the present observation of an O_2 debt is due to an incapability on the part of the lungs to increase the O_2 uptake

VIII COMMENT

The advent of jet propelled high performance aircraft introduced G time flight patterns with much longer durations of G maneuvers than had previously been technically possible After some of the mechanisms behind the phenomena of blackout and loss of consciousness experienced by aviators during headward acceleration were settled, physiologists soon became aware of a new group of problems pertaining to effects of headward acceleration Breathlessness, substernal pain and mental as well as physical fatigue became common symptoms after strenuous flight missions, especially when 100 per cent O had been supplied As a consequence, tolerance limits could no longer be expressed in terms of thresholds such as blackout or unconsciousness

Certain effects of headward acceleration on pulmonary gas exchange under air breathing have been reported in earlier papers from this laboratory The decrements originally observed in the arterial O_2 saturation were characterized by much longer time constants than those known to exist for changes in the systemic circulation, and were accordingly thought to be of interest in connection with aviation safety problems It therefore seemed worthwhile to look further into the significance of the observed changes

The present investigation has demonstrated that the arterial O_2 tension also becomes markedly and rapidly decreased under G time stresses often encountered in routine flight missions When interpreting the data presented, it should be kept in mind that the values for arterial O_2 tension in Table II and in Fig 8 represent mean values for several subjects who do not all have the same reaction patterns When analyzing biological observations the main attention is usually paid to statistical means However, when discussing tolerance limits related to safety problems, mean values are not suitable as yard sticks since due consideration must be given to the worst case This means that the individual susceptibility to gravitational stress becomes the tolerance limiting factor

In the present work the group mean of individual one minute time averages for the arterial O_2 tension was found to be 58 mm Hg in the run period (cf Table II) This value is certainly remarkably low especially when considering its implications for the O_2 supply of the brain If the lowest recorded individual values for arterial O_2 tension are also taken into consideration, these implications become more alarming As can be seen in Fig 10 in which the lowest individual 15 sec time averages of O_2 tension have been plotted seven of the nine subjects showed values which were lower than 58 mm Hg four of the subjects going below 50 mm Hg One case had a 15 sec value below 45 mm Hg

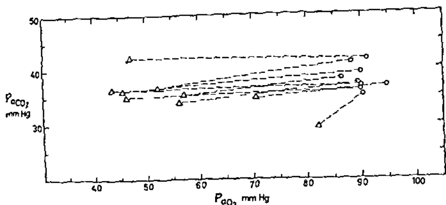
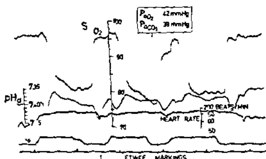


Fig 10 Maximal individ al changes in the arterial CO and O tens ons resulting from ex posur to + 5 G for 2 min plotted on the CO —O diagram *Circles* indicate ind vidual time averages over ? min prior to starting the centrifuge *Triangles* indicate lowest individual values during the course of run (time averages over 15 sec)

Fig 11 Effects of hort lasting ex posures to + 6 G (anti G su t in flated du ing runs) on arterial O saturation and pH. Arterial O and CO tens ons calculated at point of lowest O saturat on value.

Note the ap d fall of arterial O saturation in repeated exposures



It goes without saying that such decrements in arterial O_2 tensions are not compatible with undisturbed brain function even if the blood flow to the brain should be normal Moreover, the risk for deterioration of cerebral per formance might become accentuated by the rapid fall of the arterial O_2 tension the lowermost values being reached within less than one minute after the beginning of the exposure For comparison Fig 11 shows blood gas changes produced by repeated short lasting exposures to G levels some what higher than those dealt with previously in the present work The rapid decrements in the O_2 saturation and the trend towards greater rates of fall with repeated exposures are illustrated (*cf* BARR 1962)

The advent of jet propelled high performance aircraft introduced G time flight patterns with much longer durations of "G maneuvers" than had previously been technically possible After some of the mechanisms behind the phenomena of blackout and loss of consciousness experienced by aviators during headward acceleration were settled, physiologists soon became aware of a new group of problems pertaining to effects of headward acceleration Breathlessness, substernal pain and mental as well as physical fatigue became common symptoms after strenuous flight missions, especially when 100 per cent O_2 had been supplied As a consequence, tolerance limits could no longer be expressed in terms of thresholds such as blackout or unconsciousness

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IX SUMMARY

The normal postural alterations in the total and regional ventilation perfusion relationships in the lungs were exaggerated by exposing healthy subjects to prolonged hypergravitational stress in centrifuge runs (+ 5 G for 2 min with anti G suit) For evaluation of these alterations respiratory minute volume end tidal CO_2 tension and arterial O_2 saturation and pH were recorded continuously over the pre run run and post run periods

During the course of the runs expired minute volume increased from 8 6 to 20 8 l/min and "effective alveolar ventilation from 4 9 to 9 6 l/min (BTPS means of 9 subjects) The arterial pH showed a concomitant increase by 0 03 of a pH unit from a pre run value of 7 41 and the arterial O_2 tension a de crease from 90 1 to 58 0 mm Hg (calculated from recorded O_2 saturation and pH)

End tidal CO_2 tension decreased from 34 5 to 23 9 mm Hg and the arterial CO_2 tension from 38 1 to 35 5 mm Hg indicating an increase in the arterial to end tidal CO_2 difference by 8 0 mm Hg An increased arterial to end tidal CO_2 difference was thus responsible for the major part of the decrement in end tidal CO_2 tension The concomitant effective alveolar to arterial O_2 difference showed a substantial rise of 41 1 mm Hg

All dead spaces increased the alveolar dead space (= the physiological minus the anatomical dead space) by an average of 170 ml Assuming a de crease in cardiac output to 80 and 60 per cent of its pre run level, venous admixture increased to 19 8 and 15 9 per cent respectively from a pre run value of 5 8 per cent

O_2 uptake increased from a pre run value of 269 to 410 ml/min whereas CO_2 elimination increased from 216 to 391 ml/min resulting in an increment in the respiratory exchange ratio from 0 80 to 0 96

In the post run recovery period (8—10 min after the completion of the run) the arterial O_2 tension showed a residual decrease of 10 9 mm Hg as compared with the pre run level the effective alveolar to arterial O_2 difference remaining 10 5 mm Hg greater than during the pre run period

The continuous recordings of arterial O_2 saturation and pH showed that 90 per cent of the final change in arterial O_2 tension during the course of a run was completed within 60 sec The lowest individual 15 sec time average value was 43 mm Hg

The results indicate the development of gross disturbances in pulmonary gas exchange during exposure to headward acceleration The mechanisms underlying these disturbances have been discussed and may briefly be de scribed as follows

The hazards due to this type of hypoxemia must be considered more serious than a similar degree of hypoxemia induced by low O_2 pressure in the inspired air (*e g* during high altitude flying) because of the reduction in total cerebral blood flow accompanying headward acceleration at levels higher than 4 G (*cf* HENRY *et al* 1951) For, if the arterial O tension is lowered and the blood flow is already much reduced, the blood passing through the brain capillaries will soon become depleted of its oxygen to an extent that may cause loss of consciousness without being preceded by noticeable subjective warning symptoms

IX SUMMARY

The normal postural alterations in the total and regional ventilation perfusion relationships in the lungs were exaggerated by exposing healthy subjects to prolonged hypergravitational stress in centrifuge runs (+ 5 G for 2 min with anti G suit) For evaluation of these alterations, respiratory minute volume, end tidal CO_2 tension, and arterial O_2 saturation and pH were recorded continuously over the pre run run and post run periods

During the course of the runs expired minute volume increased from 8 6 to 20 8 l/min and effective alveolar ventilation from 4 9 to 9 6 l/min (BTPS, means of 9 subjects) The arterial pH showed a concomitant increase by 0 03 of a pH unit from a pre run value of 7 41 and the arterial O_2 tension a decrease from 90 1 to 58 0 mm Hg (calculated from recorded O_2 saturation and pH)

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SYMBOLS AND DEFINITIONS

The following symbols used in the text conform to standards adopted by pulmonary physiologists (Fed Proc 1950 9 602)

V	gas volume (in ml)
V_T	ventilatory volume (tidal volume) (in ml)
V_I	inspiratory volume (in ml)
V_E	expiratory volume (in ml)
V_D	dead space volume
$\dot{V}$	ventilation per unit time (in L/min)
$\dot{V}_A$	alveolar ventilation (in L/min)
$\dot{V}_D$	dead space ventilation (in L/min)
$\dot{V}_{ECO_2}$	expired volume carbon dioxide per unit time (in ml/min)
$\dot{V}_{O_2}$	volume oxygen uptake per unit time (in ml/min)
R	respiratory exchange ratio $\dot{V}_{ECO_2}/\dot{V}_{O_2}$
f	respiratory frequency (in breaths/min)
P_{ACO_2}	alveolar CO_2 tension (in mm Hg)
P_{aCO_2}	arterial CO_2 tension (in mm Hg)
P_{ICO_2}	the CO_2 tension of inspiratory gas (in mm Hg)
P_{IO_2}	the O_2 tension of inspiratory gas (in mm Hg)
$P_{\bar{v}CO_2}$	mixed venous CO_2 tension (in the lungs) (in mm Hg)
P_B	ambient atmospheric gas pressure (in mm Hg)
BTPS	body temperature and pressure saturated with water vapor
STPD	standard temperature and pressure dry (0°C 760 mm Hg)
BMR	basal metabolic rate
BSA	body surface area (in m²) (calculated according to DUBOIS & DUBOIS Arch int Med 1915 15 868)

Dash (—) above a symbol indicates a mean value Dot (·) above a symbol indicates a time derivative

In the present paper the following symbols have been used furthermore

T	threshold value of P_{ACO_2} (CO_2 threshold) indicating the point above which a ventilatory response appears when P_{ACO_2} is gradually increased
s$\dot{V}$	standard ventilation (3.5 L/min/m² BSA) This value expresses mean ventilation of all the investigated subjects during wakefulness at rest breathing (oxygen enriched) air
sT	standard threshold of P_{ACO_2} as measured at the intersection between the s$\dot{V}$ line and the rectilinear part of the ventilation P_{ACO_2} response curve The value is indicated as sT_A sT_B sT_C etc according to the EEG stage of wakefulness

SYMBOLS AND DEFINITIONS

The following symbols used in the text conform to standards adopted by pulmonary physiologists (Fed Proc 1950 9 602)

V	gas volume (in ml)
V_T	ventilatory volume (tidal volume) (in ml)
V_I	inspiratory volume (in ml)
V_E	expiratory volume (in ml)
V_D	dead space volume
$\dot{V}$	ventilation per unit time (in L/min)
$\dot{V}_A$	alveolar ventilation (in L/min)
$\dot{V}_D$	dead space ventilation (in L/min)
$\dot{V}_{ECO_2}$	expired volume carbon dioxide per unit time (in ml/min)
$\dot{V}_{O_2}$	volume oxygen uptake per unit time (in ml/min)
R	respiratory exchange ratio $\dot{V}_{ECO_2}/\dot{V}_{O_2}$
f	respiratory frequency (in breaths/min)
P_{ACO_2}	alveolar CO_2 tension (in mm Hg)
P_{aCO_2}	arterial CO_2 tension (in mm Hg)
P_{ICO_2}	the CO_2 tension of inspiratory gas (in mm Hg)
P_{IO_2}	the O_2 tension of inspiratory gas (in mm Hg)
$P_{\bar{v}CO_2}$	mixed venous CO_2 tension (in the lungs) (in mm Hg)
P_B	ambient atmospheric gas pressure (in mm Hg)
BTPS	body temperature and pressure saturated with water vapor
STPD	standard temperature and pressure dry (0°C 760 mm Hg)
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INTRODUCTION

Background

It has become increasingly clear that changes in wakefulness are normally linked to changes in several basic functions of the body. Since the introduction of electroencephalography (EEG) as a method for clinical use it has also been obvious that the EEG record mirrors wakefulness in a very characteristic way. The discovery of the reticular system of the brain stem (MORUZZI & MAGOUN 1949) and the many ensuing studies of its properties has made it likely that this system is the one mainly responsible for the coordination of EEG changes with autonomic and other bodily changes during variations in level of wakefulness (MAGOUN 1952, DELL 1952, HUGELIN 1956).

In general, however, our knowledge of the central regulation of "the cortical excitatory state" (JASPER 1936, *cf* BONVALLET *et al* 1954) and of other functional systems of the body is still very limited. Numerous data in the literature suggest a correlation between certain autonomic — such as respiratory, circulatory and muscular — fundamental functions and general changes of the EEG. Amongst these, the respiratory ones should be given a prominent position since they represent one of the most basic activities of the body.

The point of departure for the present study is the old observation that respiration changes when a person falls asleep. This phenomenon has been brought into new light by a series of recent investigations which demonstrate an anatomical overlap within the brain stem between the so called respiratory centres and the reticular system. There is also physiological evidence suggesting a close functional relationship between mechanisms regulating respiration and wakefulness. (For references, see below.)

Aim of the investigation

The primary aim of the present work is to correlate in detail in a large group of normals respiratory and EEG variables at various levels of wakefulness. Two main respiratory variables were measured: the ventilation $\dot{V}$ and the alveolar carbon dioxide tension P_{ACO_2}. The level of wakefulness was estimated with the aid of electroencephalography (EEG) in which six different levels were differentiated between deep sleep and tense alertness.

DsT difference (in mm Hg) between sT values of two different stages of wakefulness, $DsT_{A\ B}$, $DsT_{A\ C}$, $DsT_{A\ D}$ etc

SQ stimulus response quotient ("sensitivity" index), indicating the increase in ventilation per mm Hg increase in P_{ACO} at the rectilinear part of the ventilation CO_2 response curve The value is given in L/min/mm Hg/m BSA

V P point=ventilation, V, and P_{ACO_2}, expressed by the mean values from 5 consecutive breaths, when plotted in a ventilation P_{ACO} diagram

PB periodic breathing

A diagrammatic illustration of the symbols mentioned above is given in Fig 6 on page 27

For classification and symbols of the electroencephalographic (EEG) stages (levels) of wakefulness (including sleep and tense alertness) see page 21

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A characteristic, close relationship between respiration and EEG was observed early in the course of the present study, as previously reported in a brief paper (BULOW & INGVAR 1961) The findings suggested that carbon dioxide — or factors closely connected with its ventilatory action — play an important role for the changes in ventilation which accompany changes in wakefulness The role of carbon dioxide in the sleep wakefulness regulation has therefore been especially emphasized in this study Attempts have also been made to compare the findings in normals with those in diseases which involve the wakefulness function The latter results will be published in detail elsewhere (BULOW 1963 a and b) and only a brief summary is given in the present paper

Previous work on respiration during sleep

The Danish chemist SCHARLING was one of the first to investigate quantitatively changes in respiratory functions during sleep In 1843 he reported diurnal variations in the expired amount of carbon dioxide SMITH (1860) demonstrated a decreased ventilation during sleep and MOSSO (1878) and CZERNY (1892) reported differences in respiratory pattern between wakefulness and sleep Later, SHEPARD (1914) and REED & KLEITMAN (1926) observed that ventilation is very regular during deep sleep For technical reasons many of the early investigations gave contradictory results concerning the difference in degree as well as in pattern of ventilation between wakefulness and sleep

LOEWY (1890), by measuring the expired amount of CO_2 made the first evaluation of the CO_2 sensitivity of the respiratory centres He also studied this sensitivity in sleeping subjects The discovery by HALDANE & PRIESTLEY (1905) of the importance of the alveolar CO concentration for the respiratory regulation initiated several later investigations of the CO sensitivity of the respiratory centres (LINDHARD 1911, and others) STRAUB (1915) found that the alveolar CO tension (P_{ACO_2}) is higher immediately after awakening in the night than when awake in daytime From this observation he concluded that the CO_2 sensitivity of the respiratory centres is decreased during sleep in the night

In a classical study in normals MAGNUSSEN (1944) used a refined technique for measuring respiration during sleep He demonstrated changes in the expiratory pressure even during transient periods of (behavioural) sleep During continuous sleep ventilation was decreased and alveolar CO tension increased In 2 cases MAGNUSSEN studied the ventilatory response to CO_2 and found this response to be lowered during sleep From 2 experiments with spontaneous sleep ÖSTERGAARD (1944) made a similar statement when studying the respiratory effects of Evipan anesthesia

The EEG technique made it possible to measure certain general changes in the cerebral electrical activity which correlate with different levels of wakefulness (Jasper 1936, Loomis *et al.* 1937, Blake & Gerard 1937, Davis *et al.* 1938, Gibbs & Gibbs 1951 and others).

Besides the "classical" sleep stages a special state of sleep ("rapid eye movement" (REM), "activated" or "paradoxical", "rhombencephalic sleep") has been described, characterized by a low voltage fast activity in the EEG similar to that during wakefulness and furthermore by eye movements and by frequent dreaming (Dement & Kleitman 1957, Jouvet & Michel 1960, Jouvet 1962). Very recent studies of this state of sleep emphasize, however, that in man the EEG pattern is less distinctive, containing a varying amount of slower wave forms (Schwartz 1962 and others).

Mangold *et al.* (1955) found a slight increase in cerebral blood flow during sleep. They also reported that an increased arterial CO_2 tension normally preceded the onset of sleep. This gave new support to the suggestion that hypercapnia is an important sleep promoting factor (Kety 1961).

In the last few years some further studies on respiration during sleep have been reported by Robin *et al.* (1958) and by Birchfield *et al.* (1959). In contrast to Mangold *et al.* (1955) these authors did not find a definite increase in arterial CO_2 tension until the onset of sleep. The findings of Magnussen (1944) were largely confirmed. They presented experimental results from a limited number of subjects and demonstrated a marked decrease in the ventilatory response to CO ("CO_2 sensitivity") of the respiratory centres during sleep. Other recent studies of this latter problem have, however, given contradictory results. When investigating the influence of sleep on the respiratory function at low and high altitudes, Reed & Kellogg (1958) demonstrated displacement of the ventilation – CO_2 response curve (implying an increase of the "CO_2 threshold", page 51) but no certain change in the slope of the curve. With the aid of EEG Bellville *et al.* (1959) reached a similar conclusion.

The investigations quoted above have all shown that respiratory changes do take place during (or closely preceding the onset of) sleep. It has, however, not been clarified 1) how closely such changes are correlated with changes in the EEG. Furthermore 2) the question of the changes in carbon dioxide sensitivity or threshold during shifts in the level of wakefulness is still a matter of conjecture. In addition 3) the intra- and interindividual variations in respiration-wakefulness pattern have not yet been studied. These problems constitute the subject of the present investigation.

MATERIAL AND METHODS

1 *Material*

The total material consisted of all together 70 apparently healthy subjects, of whom 30 were females (aged 18—60) and 40 males (aged 18—57) Routine examination which also included chest X ray revealed no symptoms or signs of disease of the respiratory, circulatory or nervous systems

Pulmonary function was studied in all of the subjects As a rule the following variables were measured lung volumes, functional residual capacity, respiratory frequency and minute volume, maximal breathing capacity oxygen uptake, as well as the distribution of inspiratory air

The distribution of the inspiratory air was estimated by rebreathing helium in a closed system with a catapherometer The ventilatory volume necessary to get the inert gas homogenously mixed when the subject was connected to the system was measured and divided by the functional residual capacity When the qoutient obtained was below 9 it was considered normal (BÜLOW unpublished material) Occurrence of pathological ventilation perfusion relationship between different parts of the lungs was judged from this value and from the slope of the alveolar part normally a plateau of the expiratory curve of carbon dioxide concentration as measured by a capnograph (page 14) from normal and from maximal expirations (ULMER & REICHEL 1960)

In 55 of the subjects obvious changes in level of wakefulness were registered during the recording periods In 2 males however the analysis of pulmonary function revealed obvious signs of (functional) emphysema Since the measurements from these 2 persons were therefore excluded the number of subjects upon which the results were based was 53, of which 22 were females (aged 20—60) and 31 males (aged 18—49)

Routine EEG examinations were made in 51 of these 53 subjects There were no gross abnormalities found nor any focal or paroxysmal discharges The EEG controls showed a distribution similar to other normal materials such as that of GIBBS & GIBBS (1951)

In 41 cases the record showed normal conditions In 11 of these cases there was a marked tendency to drowsiness 5 additional cases demonstrated a markedly low voltage record which was otherwise not noteworthy 11 of the 41 cases demonstrated a very light diffuse or episodic admixture of 4—7 cycle / sec waves which was considered within normals limits The records of the last 5 subjects were more noteworthy S L. showed a poor alpha development and some low voltage theta episode with a slight right sided dominance S E showed a mild diffuse admixture of low voltage 3—5 cycle /sec wave A P showed some 5—7 cycle /sec episodes in the temporal region on the right side

A R showed a symmetrical record when awake but a rightsided predominance of slow waves in drowsiness I P showed bitemporal low voltage episodes of 3–6 cycles/sec waves with a leftsided dominance

In none of the cases were any EEG abnormalities observed during sleep

A detailed analysis of the subjects of the present study including further EEG investigations is at present in progress (BULOW & INGVAR 1963 c)

The above mentioned pulmonary function tests were made in all the 53 subjects with the exception of 3 subjects which, however showed a normal slope (plateau) of the alveolar part of the end expiratory CO_2 curve during capnography and also normal vital and maximal breathing capacities

In 4 subjects (M L A A I F and Y T) the maximal breathing capacity was reduced by somewhat more than 20 per cent of predicted normal value in 5 of the subjects (P B I P I F L K and K B) the residual volume in per cent of the total lung capacity exceeded the predicted normal value by slightly more than 6 units

In 2 subjects (Y T and Y R) the difference between mid expiratory and maximal expiratory P_{CO_2} by capnography slightly exceeded 6 mm Hg During spontaneous breathing at rest however there were distinct end expiratory plateaux In 1 subject (I D) the index estimated from the helium distribution was 9 7 in all other subjects the value was below 9

Of the 53 subjects who slept during the recording periods all except 3 (I O A H and S I) were examined shortly after they had left off work in the morning when they otherwise went to bed 37 of them were used to night work for more than a year usually without any periods of day work The remaining 13 subjects (I O A H and S I not included) were occasionally on night work (mostly hospital staff, medical students members of the armed forces telephone operators and cab drivers) I O A H and S I had not had any night work

Several of the subjects were studied repeatedly in the morning after night work as well as after several consecutive nights of sleep 6 subjects were studied on one occasion in the morning and on another occasion during the night 6 subjects were investigated in the afternoon

2 *Recording equipment*

The investigations were carried out in a dark quiet room with the subject in a comfortable supine position (Fig 1) The carbon dioxide content of the respiratory air the ventilation and the EEG were recorded continuously The main recording equipment consisted of a *spirometer* (INSTRUMENTA Lund) modified for the purpose of the investigation an *infra red light absorption gas analyzer* which continuously recorded the carbon dioxide content of the ventilatory air (CAPNOGRAPH GODARD type CG/38003 coupled to a direct writer OMNIASCRIPTOR GODARD) as well as a *four channel* ELEMA MINGOGRAPH *recorder* (Type M 42 B) including EEG-

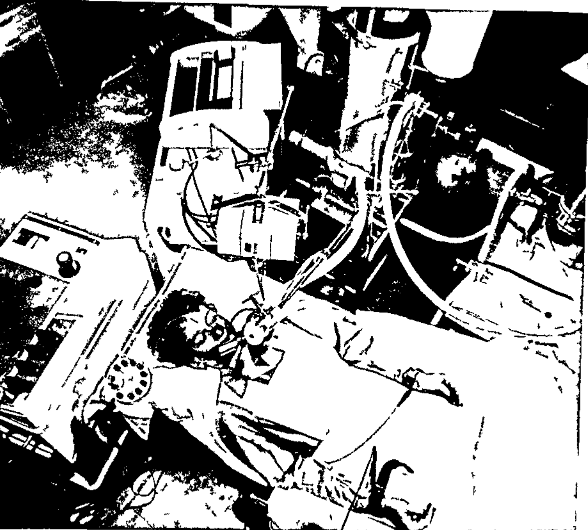
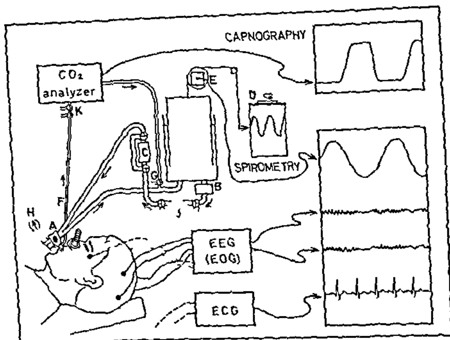


Fig 1 Experimental arrangements (To the left the Mingograph and above the Capnograph and the spirometer)

A schematic drawing of the recording arrangements is given in Fig 2 Letters in the following section refer to Fig 2

Via a four way cock (A) close to the mouthpiece oxygen enriched air was conducted via a pump (B) and a soda lime absorber of carbon dioxide (C) round the closed spirometric system The variations in the volume were recorded directly by a three speed (30 300 and 3000 mm/sec) kymograph (D) and usually also on the same paper as the EEG on the MINGOGRAPH by means of resistance variations in a potentiometer (F) mounted on a moving part of the spirometer A short tube (F) conducted a small portion of the respiratory gas which was sucked continuously from the oral cavity through the mouthpiece to the CO_2 analyzer cell This gas was then conducted via the pump of the capnograph back into the spirometric system via a small three way cock (G) With the subject connected the capnograph was thus shunted to that part of the rebreathing circuit which conducted the respiratory gases from the subject to the spirometer bell Variations in the carbon dioxide content



Fi ? *Princ pal recordi g equipment* See text on this page

were recorded by the OMNIASCRIPTOR and also often on one of the channels of the MINGOGRAPH on the same paper as the EEG and the ventilation The EEG was recorded by conventional technique from 4 electrod s placed parieto occipitally according to the international sy tem (positions left si le C? D1 r ght de C4 D?) (JASPER 19?8)

In some cases the movements of the eyes were also recorded on the Mingograph from two leads placed at the outer angles of the eyes (electro-oculography) In addition in many c es the electrocardiogram was recorded from one of the conventional body lead

a Spi om ter The follow ng parts were constructed e pecially for the examinat on

1) A four way cock (A) wa mounted which could be manipulated at a d stance without the subj ct notici g anything ?) Th e in trumental dead space including that of the mouth p ece was educed to ?0 ml 3) The inlet for in piratory air could be switched from the pirometric system or f om room air to a KROGH valve (modified according to VON DOBELN 1949) which was integrally attached to the cock and which only ga e a negligible add tion of dead space The expiratory ai could by this arran ement eas ly be collected in another pirometer (H) for determination of the CO_2 outp t (u ually for 30 seconds to one minut) For dete m nation of the mixed venous carbon dioxide ten ion (P_{CO_2}) the re piratory air could by the four way cock (A) al o be connected to a rubber bag in wh ch exp ed air was rel reathed

4) The circulat ng re piratory gas in the sp rometer could readily be shunted past the carbon d ox de filter (C) by means of ? three way cocks with consequent accumulation of the exp red carbon dioxide in the system Usually an extra volume of about 20 L was then conne ted to the sy tem 5) This added volume could readily be di connected by

2 other three way cocks (J) so that the circulating respiratory air could be distributed *along two loops connected in parallel* one of which conducted the gas through a eparate CO filter The ordinary filter was meanwhile removed from the circuit By manual regulation of the flow through the loop with the CO filter the CO_2 content in the system could be maintained at different levels between 0 and 3 per cent The CO_2 content of the in piratory air was then checked visually on the direct writer If necessary CO_2 was added to the system from a steel tube via a reducing valve and a flow meter From another tube pure oxygen was conducted continuously through a flow meter (ROTAMETER CROYDON) to compensate for the amount consumed by the subject A constant oxygen content in the spirometer was maintained by adjusting the flow so that the tracing of the end tidal air level on the kymograph was horizontal The rotameter was calibrated with an accuracy of $\pm$ 10 ml O /min by the manufacturers (ROTAMETER Manufact Ltd CROYDON)

The spirometer bell was balanced *in all positions It was made of thin (0 3 mm) copper* and suspended on the spirometer stand with a chain over the pulley which was mounted *on ball bearings The potentiometer (E Fig 2) was mounted on the axis of this pulley* It had a negligibly small friction resistance

Calibration of spirometer The temperature of the gas in the spirometer bell which varied between 26° 0 and 29° 0 C was read intermittently during each examination The temperature increa ed somewhat every time the CO absorber was re connected in the circuit after the CO_2 had been collected in the sy tem All respiratory volumes measured were corrected to BTPS (body temperature atmospheric pressure saturated with water vapour) The ambient atmospheric pre sure was read from a barometer calibrated at regular intervals

The respiratory volumes were read from the spirographic paper on which 5 mm deflexion corresponded to 100 ml The apparatus was calibrated with small known volumes of gas The amount of deflexion per 100 ml was the same irrespective of the start level in the spirometer bell The spirometer was also checked with the aid of an electric pump for stroke volumes (tidal air) of 250 to 900 ml and ventilatory frequencies of 10—20 per minute

The air flow resistance measured at the site of the connection of the spirometer to the mouthpiece was negligibly small (pressure variations le s than $\pm$ 3 mm water) even for *such high respiratory frequencies* as up to 20 breaths/minute and for tidal volumes of up to 1 liter (*i e* at the upper limits reached under the conditions used in the present *investigation)*

The rate of the spirometer paper was calibrated and correction factors for changes in *the rate were calculated to permit an accuracy of $\pm$ 0 15 sec through the entire course* of the registration

The constant gas flow *independent of respiratory movements through the* shunted capnograph was low 1 2 L/min Thus even when the differences between inspiratory and expiratory flow were substantial the effect of the shunt flow on the shape of the spirographic recording of the breath could be ignored

b *Capnograph* The volume of the analyzer cell of the capnograph was *0 4 ml and* the volume of the 40 cm long tube (inside diameter 3 mm) between the mouthpiece and the analyzer was 2 8 ml The flow rate of 1 2 L/min was adjusted *by means of a flow meter* which was repeatedly calibrated

With the aid of two small three way cocks (K) (with the same *inner diameter* as the supply tube) the analyzer could easily be switched over for calibration with different test gases or for connection with the extra spirometer (for *measurement of mean* $\bar{\imath}$ CO_2 of expired air) Turning the large four way cock at the mouthpiece (A) the flow of gas

from the capno raph hunt could also be conducted either to the atmo pheric air to the main spirometer or to the extra spirometer via the cock (G) near the spirometer bell

The highe t value noted for the plateau of the end exp ratory carbon diox de content was read from the thermosen ble recording paper of the Omniascriptor and mea ured as percenta e alveolar carbon d oxide This value was expressed in the conventional way in mm Hg by multiplyin, with the ambient atmo pheric pre sure corrected for water apour (P_B 47)

The re pon e time of the capnograph and the d rect writer (90 per cent deflex on) for a momentary change from 0 per cent to 5 per cent of carbon d oxide at the moutl p ece was found to be 0 3 ec with the flow rate used i e 1 2 L/min

Cal bration of capno raph and d rect writer The capnograph was calibrated repeatedly again t 4 to 6 gas mixtures contain ng N_2 and different amounts (2—7 5 per cent) of CO_2 in 40 per cent O_2 The e mixtures were analy ed by the micro-Scholander technique (SCHOLANDER 1947) Within the measuring range of 30 to 45 mm Hg the accuracy of the capno raph was ± 0 5 mm Hg The drift of the capnograph and of the writer was low together at most ± 0 mm Hg per hour By means of repeated calibrations during the examination the ba e line a well as the deflection accuracy in the measuring range was readjusted Hence the error from the drift was substantially compen ated and the accuracy wa improved to ± 0 3 mm Hg within the mea uring range of 30—45 mm Hg

A small con tant de iation from linearity of the Omniascriptor recording — a slight S hape — was noted and its effect on the mea uring values was eliminated The records were read (under a magnifying glass) with an accuracy of ± 0 2 mm Hg

e EEG ampl fier The frequency re ponse of the EEG amplifier was linear from 1 5 up to 60 cycle /sec The sen tivity was 20 μ V/cm In general an amplification of 50 μ V/cm was used for the records The amplifier had a time constant of 0 1 sec which permitted record ng of rap d ocular movements by electro-oculography (EOG)

The *Mingograph* had a l near frequency response up to 700 cycles/sec Its amplitude linearity was ± 0 mm its base line linearity ± 1 mm In most experiments the EEG was recorded in two of the channels and the capnograph record and/or ventilation alternating with electrocardiography n the remaining two By simultaneous recording of the EEG capno ram and entilation on the same paper it was always poss ble to achieve a close analy is of the time relationship between the e three variables The records traced by the omnia criptor and the piromete were correlated with the Mingograph record and each breath wa gi e a number on all records

At som examin tions oculog aphy and EEG (each through 2 channels) were done also on an extra EEG machin to secure more space for recording the sequences of other events on the M ngograph

3 Examination procedure

Preparat on of subject All of the subjects to be exam ned were requested to abstain from the u e of all dru for 2 days before the examination None of them was accu tomed to hypnotics All the subject were informed that the remuneration given for participation (Sw Cr 35 —) would be granted even if they could not sleep during the examination As an extra precaut on it was alleged that any intake of dru s would be revealed by the EEG Stimulating be ra es (alcoh lic drinks coffee or tea) and smoking were forbidden for 8 hour before the examination All the subjects had a li ht meal rich in carbohydrates 3 to 4 hours before the beginning of the recordin, period

B fore the recordin was started the subjects were allowed to rest comfortably during at lea t 40 minutes in the supine po ition with a pillow under the head on the examination

2 other three way cocks (J) o that the circulating re piratory air could be di tributed along *two loops connected in parallel* one of which conducted the gas through a separate CO_2 filter The ordinary filter was meanwhile removed from the circuit By manual regulation of the flow through the loop with the CO_2 filter the CO_2 content in the system could be maintained at different levels between 0 and 3 per cent The CO_2 content of the in piratory air was then checked visually on the direct writer If nece ary CO_2 was added to the system from a steel tube via a reducing valve and a flow meter From another tube pure oxygen was conducted continuously through a flow meter (ROTAMETER CROYDON) to compensate for the amount consumed by the subject A constant oxygen content in the spirometer was maintained by adjusting the flow so that the tracing of the end tidal air level on the kymograph was horizontal The rotameter was calibrated with an accuracy of $\pm$ 10 ml O_2/min by the manufacturers (ROTAMETER Manufact Ltd CROYDON)

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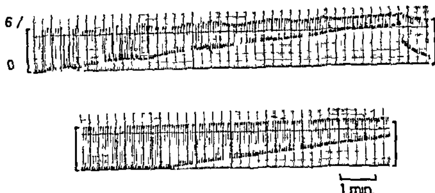


Fig 3 Subj B B male aged 23 Capnographic record of two CO_2 tests *Above* Typical periodic breathing (PB) at the beginning gradually disappearing towards the end of the test *Below* 15 min later The subject is in profound stable sleep (EEG stage D) Very even breathing whithout any signs of periodicity

was measured repeatedly by letting the subject rebreathe the expired carbon dioxide in the closed system of 30 L volume (page 13) For convenience this measurement will be called CO_2 test

A CO_2 test was started without the subject being aware of it by conducting the circulating gas in the system through the shunt past the CO_2 absorber (C Fig 2) There occurred a gradual increase of the inspiratory as well as the alveolar CO_2 tension (Fig 3) After about 10 minutes when the CO_2 content of the inspiratory air reached about 5 per cent the CO_2 filter was replaced into the circuit and the accumulated CO eliminated In order to restore a reasonable respiratory steady state the time interval between the CO_2 tests was as a rule more than 10 minutes

$\dot{V}_{ECO_2}$ $\dot{V}_{O_2}$ $\dot{V}_A$ *and* V_D In addition to the three main variables ventilation P_{ACO_2} and EEG determinations were made of the $\dot{V}_{ECO_2}$ $\dot{V}_{O_2}$ $\dot{V}_A$ and V_D repeatedly and at different levels of wakefulness

$\dot{V}_{ECO_2}$ (the CO_2 output) was calculated when expiratory air was collected and measured in the exta spirometer (II Fig 2) the fan of which mixed the gas homogeneously A small portion was tapped for a few seconds through the capnograph for determination of the CO concentration From $\dot{V}_{ECO_2}$ and prevailing P_{ACO_2} the $\dot{V}_A$ and V_D were calculated according to the usual formulae

$$\dot{V}_A = \dot{V}_{ECO_2} \; k/P_{ACO_2} \tag{1}$$

and

$$V_D = (\dot{V}_T - \dot{V}_A)/f \tag{2}$$

where f = respiratory frequency/min and k is a constant (Symbols see page 5) In the calculation of V_D the instrumental dead space (20 ml) was subtracted

$\dot{V}_{O_2}$ (the oxygen uptake) was determined closely before or after the $\dot{V}_{ECO_2}$ from the slope of the spirogram after the supply of oxygen to the spirometer had been shut off

couch The subjects were not allowed to lie on the side in order to avoid the effect of change in posture on the distribution of ventilation and perfusion in the lungs (SVANBERG 1957)

Mouthpiece A well fitting mouthpiece as well as a nose clip were selected Anaesthetic ointment (Xylocain Ointment 5 per cent) was applied in and around the nostrils Care was taken that the mouthpiece and the spirometer connection were in proper position and that the mouthpiece fitted well between the lips and the teeth so that it would remain in proper position even when the tone of the facial muscles and chin decreased during sleep The position of the body and of the head contributed to prevent the lower jaw from falling as much as it would in the sitting posture and thereby decreased the risk of leakage

Leakage In some cases leakage occurred while the subject was sleeping and then generally through the nose When leakage occurred it always did so at first during expirations only It could be readily detected on the spirogram because the horizontal tidal level began to rise when the spirometric volume decreased during each expiration During such leakage it was seldom necessary to touch the sleeping subject or to interrupt the recording The decrease in volume in the spirometer was then compensated for by continuous supply of air oxygen mixture Measurements of the ventilatory volumes were always done from the inspirations (see below) Therefore when the leakage was compensated the volume of gas added during each inspiration (usually less than 0 3 L/min) was subtracted from the inspiratory volumes measured In the event of leakage the tightness of the system during the inspiration was tested by disconnecting the carbon dioxide absorber for a short time Then some CO_2 was collected in the system If there was an admixture of room air to the inspired gas this gave a short downgoing spike on the capnograph record during the very first part of the expiration (during which pure dead space air from the subject was measured) Leakage very rarely occurred during inspiration and when it did it was usually due to the mouthpiece having slipped out of the mouth No ventilatory calculations were made from tracings during inspiratory leakage

Recording procedure The examinations usually lasted 4 to 6 hours, during which the subject s eyes were closed The air within the closed re breathing system contained 40 per cent oxygen This prevented stimulation of ventilation by hypoxia

During the beginning of the recording period the subject was usually awake There then followed a period with signs of increasing drowsiness in the EEG In most cases this period was followed sooner or later by sleep sometimes however not until after some hours In some cases uninterrupted periods of sleep could be recorded for more than one hour, though usually of somewhat shorter duration The level of wakefulness varied several times during the examination Periods of varying duration of deep and of light sleep changed with drowsiness and wakefulness Towards the end of the recording period the sleeping subjects usually woke up spontaneously and remained awake In several cases recording was also made for a longer period while the subjects were in an alert state

"CO_2 tests' During different stages of wakefulness, as monitored in the EEG, the ventilatory response to increased alveolar carbon dioxide tensions

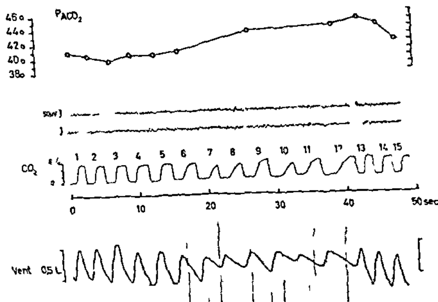


Fig. 4 Subj L. H male aged 22 From above Measured values of (end plateau) P_{ACO_2} EEG capnographic and pirographic records (all on the same scale) At expirations Nos 7 8 10 and 11 no end plateau is reached on the capnographic record At Nos 9 and 12 however a short plateau is recognizable From the upper tracing it is evident that these P_{ACO_2} values are reliable The large expirat on 13 following an only small inspiration (of the same order as in No 12 about 300 ml) shows only slight further increase in P_{ACO_2} measured (At the beginning and end of the record there are typical end expiratory plateaux)

for determining P_{ACO_2}) On the basis of calculated normal relations between alveolar and tidal volumes respiratory dead space being at least 100—125 ml the following criteria for acceptable end expiratory P_{ACO_2} values were used

a) V_I exceeds 3/4 of the average tidal volume for the subject and is not less than 300 ml V_E is not less than V_I

b) When V_E is high (at least 1/3 higher than the mean tidal volume) it is accepted even when slightly less than V_I (This difference may be greater when V_I is greater but never more than 100—150 ml.)

c) When both V_I and V_E have fallen below 300 ml for several consecutive breaths the end expiratory P_{CO_2} is again accepted if it reaches a (short) plateau.

5 *Classification of level of wakefulness*

With the exception of the states of tense alertness and REM sleep (see below) the various levels of wakefulness were classified *entirely from the EEG record* In general stages A to E according to the system of LOOMIS *et al* (1937) and with the criteria given by GIBBS & GIBBS (1951)

for some minutes The fall (a few per cent) in the oxygen content of the system was compensated immediately afterwards by increased addition of oxygen

4 *Relationship between P_{CO_2} measured as expiratory end plateau gas tension and "ideal" alveolar P_{CO_2}*

In general the presence of an end expiratory plateau was considered a *prerequisite for the measurement* by capnography of the P_{ACO_2} of a single breath The peak of the end plateau corresponds closely to the mean alveolar P_{CO_2}, provided that the ventilation perfusion relationships of the lungs are normal and that there is no abnormal blood shunting past the pulmonary alveoli (COLLIER *et al* 1955, GOPFERT & HENNEBERG 1956)

Conditions for an approximate agreement between the alveolar and arterial P_{CO_2} are normally favourable in the supine position when the *perfusion of the blood is more evenly distributed to the different parts of* the lungs Sometimes, especially when the subject was falling asleep, transitory changes were seen in the ventilatory pattern with a very low tidal air and hence no end expiratory plateaux were recorded Sometimes even when the respiratory volumes were high the end expiratory plateau was missing owing to changes in rate of expiration On the other hand, a false plateau was sometimes observed when the expiration was inter*rupted before reaching the end tidal level Such false plateaux were excluded* from the calculations (see below)

It has long been known (KROGH & LINDHARD *1913*) *that* the respiratory volume V_E should be more than twice as large as V_D for the end expiratory portion to contain representative alveolar air However V_D may decrease somewhat when V_T falls to a lower level such as during sleep (see page 00) The close dependance of the measured P_{ACO} upon the degree of ventilation *(measured by the aid of the inspiratory volume)* was always apparent on breath by breath comparison (Fig 1) between the ventilation *(V) and the end expiratory P_{CO} (V per breath is expressed as V_I/t where* t gives the duration of the breath, from the beginning of inspiration until the beginning of the following inspiration) Provided that the end tidal level is largely stable, the end expiratory P_{ACO} always varies inversely to the V

When re piratory volume falls suddenly to less than about 300 ml (*i e* to a value which is less than twice the ordinary dead space V_D) the expiratory CO_2 curve will however *not reach an alveolar plateau during the first breaths after the decrea e in volume* During these breaths however the end expiratory P_{CO_2} increa es markedly and often reaches a short end plateau (Fig 4) The P_{CO_2} values then follow the variations in ventilation in a normal (inverse) way breath by breath This was usually ob erved provided that the re piratory volume was larger than about 250 ml (If the re piratory volumes were smaller there would usually be no alveolar plateau and such breaths could therefore not be u ed

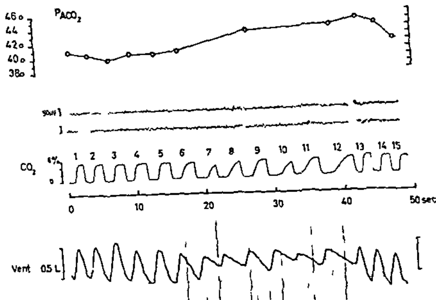


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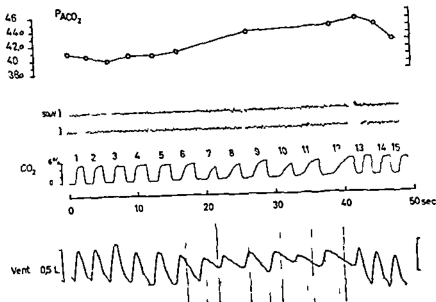


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were used (They imply a modification of the border between stages A and B) The five stages are illustrated in Fig 5

Stage A Wakefulness The record was dominated by modulated alpha activity (25—75 μV in parieto occipital leads 8—12 cycles/sec) without any appreciable admixture of lower frequencies This corre ponds to wake fulne s at rest with closed eyes

Stage B Drowsiness The background activity is of smaller amplitude with episodes of a more continuous activity of frequencies from the theta band (4—7 cycles/sec) The alpha activity is only sporadic or completely absent *Stage B_1* was introduced in a few cases in which no clearcut stage B was ob erved B_1 repre ents an intermediate stage between A and B since it contained some alpha activity which however was clearly reduced in voltage and amount as compared with stage A and did not exceed one third of the EEG period measured

Stage C Light sleep There is usually a distinct tendency to 'sleep spind les of 12—14 cycles/ ec The background activity is dominated by theta waves usually of somewhat lower frequencies than in stage B and a moderate occurrence of delta waves The amplitudes are greater than in stage B If sleep is very light biparietal bumps may occur This stage is also characterized by the appearance of K complexes

Stage D Deep sleep There is mainly delta activity of 1—3 cycles/sec of larger amplitude than in stage C Sparse spindles are also seen which are of lower frequency than in stage C

Stage E Very deep sleep The EEG is dominated by high voltage slow delta waves with frequencies of often le s than one cycle/sec

Rapid eye movement sleep (REM sleep) is usually characterized by frequent and rapid movements of the eyes recognized on the electro oculogram and by a low voltage fast back ground activity in EEG (DEMENT & KLEITMAN 1957 JOUVET & MICHEL 1960) Usually however a di tinct tendency to somewhat slower waves in the theta band are ob erved (SCHWARZ 1962 OSWALD 1962) This stage can therefore often resemble stage B in the EEG and sometimes even a stage between B and C

In a certain number of subjects who were already wide awake and with an alpha pattern in the EEG a condition of *tense alertness* here called stage A_a was obtained in one of the following ways

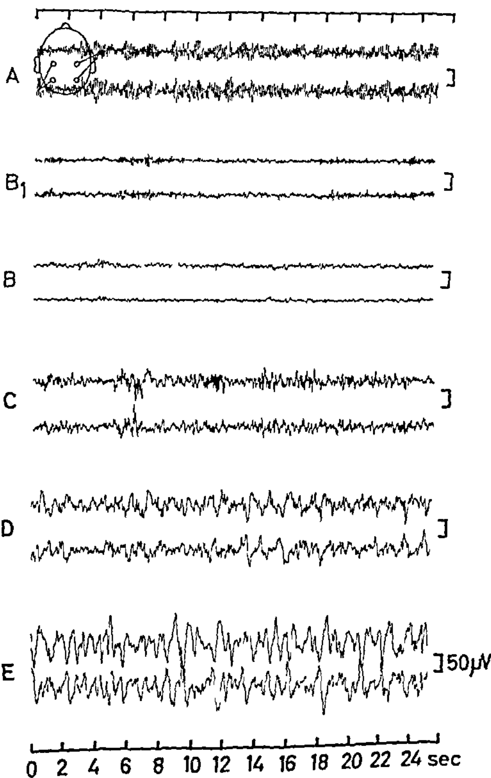


Fig 5 Typical EEG stages of wakefulness (in one subject S I male aged 21) For classification and criteria see text

(40 determinations) was 3 2 (± 0 2) mm Hg when the CO_2 content of inspiratory air was below 2 5 per cent There was no change in this a—A difference between wakefulness and leep Nor was there any change in this difference when the values were separated according to the size of the tidal volume into two groups of equal size or between samples with high and low P_{aCO_2} when instead the values were eparated according to P_{aCO} in equally sized groups Neither did any obvious change occur in this difference between P_{aCO_2} and P_{ACO_2} during arou al reactions For the later part of the CO_2 test when the in piratory air contained 2 5 to 5 0 per cent CO_2 the difference between P_{aCO_2} and P_{ACO_2} was slightly reduced to 2 5 (± 0 3) mm Hg (14 determintions) Thus the mean decrease of this a—A difference was 0 7 mm Hg

The pH varied largely inversely with the P_{aCO_2} while the standard bicarbonate did not vary with the P_{aCO}

$P_{\bar{v}CO_2}$ The mixed venous CO_2 ten ion ($P_{\bar{v}CO_2}$) was repeatedly measured with the capnograph in 16 studies on 15 subjects by means of rebreathing for 30 to 40 seconds in a rubber bag of about 1 L volume containing somewhat more than 7 per cent CO in O_2 (COLLIER 1956) Within 15—20 seconds a plateau was reached Then the CO_2 ten ion of the inspiratory air was the same as that of the expiratory air This plateau value indicated the (oxygenated) $P_{\bar{v}CO_2}$ For the calculation of P_{aCO} by this indirect method of measurement 6 mm Hg was subtracted from the values obtained during a respiratory steady state (COLLIER 1956 CAMPBELL & HOWELL 1959)

The results are given in Table II The mean difference between the calcu lated P_{aCO_2} and the P_{ACO} measured half a minute before this CO_2 rebreathing was 3 0 (± 0 1) mm Hg

Both control methods indicated that the expiratory end plateau value measured was about 3 mm Hg lower than the P_{CO_2} of the arterial blood The interindividual variations found by both methods were roughly of the same magnitude The scatter around the mean a—A differences found was somewhat wider when the direct arterial control method was used However two quite different methods were here compared with each other The error of the direct measurement on the arterial blood (SIGGAARD ANDERSEN et al 1960) might be responsible for a large part of this catter

It should be stressed that the e are no reasons to believe that the mean difference of about 3 mm is not due mainly to methodological differences since a lot of data from literatu e support th as umption that arterial and mean alveolar P_{CO_2} are approximately ident cal in normals

a) The subject who had his eyes open was repeatedly requested to concentrate sharply on some problems or interesting recent incidents At the same time he was repeatedly disturbed by auditory and visual impulses of most varying type, intensity and frequency

b) The subject was repeatedly given mental tasks to perform either arithmetic problems or a so called Sterzinger test (i e a long series of random combinations of letters of which certain constellations should be crossed out)

6 *Controls*

Alveolo arterial CO_2 tension

P_{aCO_2} The arterial carbon dioxide tension (P_{aCO}) as well as pH and standard bicarbonate were measured by the micromethod according to SIGGAARD ANDERSEN *et al* (1960) in 5 subjects, whose EEG showed periods of alertness and of sleep Several arterial samples from an indwelling catheter in one brachial artery were taken during the state awake, during sleep, as well as during the period of sleep onset when there was marked periodic breathing (page 46) The P_{aCO_2} was also measured at the moments when the EEG showed typical arousal reactions, and during CO_2 tests The P_{aCO} values were correlated with the end expiratory P_{ACO_2} values measured approximately 10 seconds earlier, i e a period roughly equal to the circulation time from the lungs to the peripheral artery

The results are given in Table I The mean difference $P_{aCO_2} - P_{ACO}$

Table I *Differences between arterial and alveolar (end expiratory)* P_{CO_2}
(Within brackets the number of determinations)

Subj	Level of wakefulness			Tidal volume (V_T)		Arterial CO_2 tension	
	Awake	Drowsy	Asleep	Low	High	Low	High
B H III	(2) 2 8		(4) 3 2	(2) 3 5	(4) 2 8	(3) 2 5	(3) 3 6
K A N II	(5) 4 4	(1) 5 1	(4) 3 3	(5) 4 0	(5) 4 1	(5) 4 1	(5) 4 0
K N I	(3) 1 9	(2) 1 3	(3) 2 2	(4) 1 6	(4) 2 1	(4) 1 5	(4) 2 2
V A II	(2) 2 1	(3) 2 4	(3) 2 9	(4) 2 6	(4) 2 4	(4) 2 0	(4) 3 1
K B II	(2) 4 6	(3) 3 7	(3) 4 4	(4) 4 0	(4) 4 4	(4) 4 3	(4) 4 1
mean	3.2	3 1	3 2	3 1	3 2	2 9	3 4

Oxygen content of inspiratory gas and of arterial blood

In order to ensure that the oxygen content of the inspiratory air was maintained during the recording periods the spirometric system was checked for any leakage before and after every examination This was often done by adding carbon d oxide to the spirometer with the fan on and the CO_2 absorber disconnected The carbon dioxide content was found to be stable after several hours checking with capnography In some of the examinations during sleep helium was add d The content of this gas was followed continuously on a CAMBRIDGE catapherometer A very slight decrease (less than 1 per cent) occurred in the course of a few hours

Oxygen saturation of arterialized capillary blood was followed continuously photometrically with an oximeter (KIFA Stockholm type BERG B3) with an ear piece according to MILLIKAN (1942) in ten examinations mainly on subjects who showed marked periodic respiration during decreased wakefulness often with periods of apnoea The arterial blood was considered saturated with oxygen during in piration of 40 per cent oxygen since further increase in the oxygen content produced no further increase of the deflexion of the oximeter The variations in oxygen saturation never exceeded 2 to 3 per cent not even during the widest variations in ventilation including periods of apnoea

7 *Calculation of the ventilatory response to carbon dioxide*

a *Time delay between a transient change in P_{ACO_2} and in ventilation*

This delay was measured in two different ways

1 The subjects inspired 7 per cent CO_2 for about 40 seconds The time was measured between the first inspiration following the end of the CO_2 rebreathing and the moment when a definite decrease in ventilation was registered The time lag varied between 21—27 econds (Table III left)

2 The subjects breathed 7 to 14 per cent CO_2 for 2 or 3 breaths and the time lag to maximal ventilatory increase was measured This time lag varied somewhat more between 18—28 seconds (Table III right) The ventilatory changes were here small and it was sometimes not possible to define the maximal ventilation re ponse to a CO_2 stimulus *from spontaneous* variations in single breaths This was usually the case when air mixtures with a CO_2 content of le s than 5 per cent were used

The results obtained by both methods suggest that when a sudden or transient change in alveolar carbon dioxide pressure causes a ventilatory response its maximum can be recorded after about 25 seconds

b *Correlation of level of wakefulness ventilation* (V) *and P_{ACO_2}*

For these correlations periods of 5 *con ecutive* breaths were used V and P_{ACO_2} were measured from each of the 5 breaths and their mean values *gave a V—P point* in a V—P diagram *V was always calculated as the quotient between inspiratory volume and duration of the breath* Every V value was divided by the body surface area (BSA) of the subject

Table II *Differences between* $P_{\bar{v}CO_2}-6$ *as measured by* CO_2 *rebreathing and alveolar (end expiratory)* P_{CO}

Subj	$[P_{\bar{v}CO}-6]$ (above) $[P_{\bar{v}CO_2}-6]-P_{ACO_2}$ (below) I	II	III	IV	V	mean
I O	38 5	39 3	40 2			
	3 3	*3 3*	*3 4*			3 3
I F	37 4	37 4	42 1	40 2		
	3 2	*3 2*	*2 9*	*3 4*		3 2
S F	39 4	38 0	37 5	38 5	36 7	
	3 0	*3 0*	*3 0*	*3 0*	*3 1*	3 0
T S	39 2	40 1				
	2 7	*2 4*				2 6
F N I	39 1	39 1				
	3 3	*4 4*				3 9
L K	40 6	41 5				
	2 8	*3 1*				3 0
L H	38 8	37 3				
	2 7	*2 2*				2 5
I S	40 3	40 3				
	3 1	*3 2*				3 2
K B I	40 8	40 8				
	1 8	*2 1*				2 0
V R	40 4	39 1				
	4 0	*3 7*				3 9
B H III	37 1	36 7	38 1			
	2 3	*2 8*	*2 9*			2 7
K Å N II	41 4	40 9	40 5			
	3 3	*3 0*	*2 9*			3 1
K N I	33 8	34 3	32 3			
	2 9	*3 5*	*3 2*			3 2
V A II	40 1	36 9				
	2 4	*3 6*				3 0
K B II	42 1	43 0	42 1	40 2		
	2 6	*2 3*	*2 1*	*3 5*		2 6
R T	35 5	35 9	35 9	33 6	34 4	
	2 9	*2 7*	*3 1*	*3 2*	*3 2*	3 0

In the foregoing principal observations were presented (page 18) which show that the expiratory end plateau measurement (under the defined conditions) sensitively reflects the variations in alveolar P_{ACO} The controls here reported render further evidence

The results support the conclusion that breath by breath variations in expiratory end plateau P_{ACO_2} *satisfactorily reproduce corresponding changes in the arterial blood*

Oxygen content of inspiratory gas and of arterial blood

In order to ensure that the oxygen content of the inspiratory air was maintained during the recording periods the spirometric system was checked for any l akage before and after eve y examinat on This was often done by adding carbon dioxide to the spirometer with the fan on and the CO_2 absorber disconnected The carbon dioxide content was found to be stable after several hours checking with capnography In some of the examinations during sleep helium was added The content of this gas was followed continuously on a CAMBRIDGE catapherometer A very sli ht decrea e (less than 1 per cent) occurred in the course of a few hours

Oxygen saturation of arterial zed capillary blood was followed continuou ly photometri cally with an oximet r (KIFA Stockholm type BERG B3) with an ear piece according to MILLIGAN (194) in ten examinations mainly on subjects who showed marked periodic respiration during decreased wakefulness often with periods of apnoea The arterial blood was considered saturated with oxygen during in piration of 40 per cent oxygen since further increase in the oxygen content produced no further increa e of the deflexion of the oximeter Th e variati ns in oxygen saturation never exceeded ? to 3 per cent not even during the widest variations in ventilation including periods of apnoea

7 Calculation of the ventilatory response to carbon dioxide

a Time delay between a transient change in P_{ACO_2} and in ventilation

This delay was measured in two different ways

1 The subjects inspired 7 per cent CO_2 for about 40 seconds The time was measured between the first inspiration following the end of the CO_2 rebreathing and the moment when a definite decrease in ventilation was registered The time lag varied between 21—27 seconds (Table III left)

2 The subjects breathed 7 to 14 per cent CO_2 for 2 or 3 breaths and the time lag to maximal ventilatory increase was measured This time lag varied somewhat more between 18—28 seconds (Table III right) The ventilatory changes were here small and it was sometimes not possible to define the maximal ventilation re pon e to a CO_2 stimulus from spontaneous variations in ingle breaths This was usually the case when air mixtures with a CO_2 content of less than 5 per cent were used

The results obtained by both methods suggest that when a sudden or tran ient change in alveolar carbon dioxide pressure causes a ventilatory re pon e its maximum can be recorded after about 25 seconds

b Correlation of level of wakefulness ventilation ($\dot{V}$) and P_{ACO_2}

For these correlations periods of 5 consecutive breaths were used $\dot{V}$ and P_{ACO_2} were measured from each of the 5 breaths and their mean values gave a $\dot{V}-P$ *point in a* $\dot{V}-P$ *diagram* $\dot{V}$ *was always calculated as the quotient between inspiratory volume and duration of the breath* Every $\dot{V}$ value was divided by the body surface area (BSA) of the subject

Table III *Time delay for a ventilatory response to a transient change in* P_{ACO_2}
(Within brackets the number of determinations)

A		B	
Subject	From end of 40 sec CO_2 rebreathing	Subject	From onset of 2 or 3 single breaths of CO_2 rebreathing
H F	(6) *22 1*	F A	(2) *20 8*
E H	(4) *26 3*	E W	(2) *18 3*
E A	(3) *25 4*	K B B	(2) *27 6*
K B I	(4) *26 0*	A K B	(2) *26 5*
K B II	(4) *26 8*	I Sd	(4) *17 4*
V R	(1) *22 5*	R T	(3) *25 8*
B H III	(3) *20 7*		mean *22 7*
K A N II	(3) *25 9*		
K N I	(3) *22 5*		
A H II	(3) *24 2*		
I S	(2) *26 8*		
S I H	(3) *22 3*		
	mean *24 3*		

According to the above mentioned time delay, P_{ACO} was estimated from the five breaths occurring approximately 25 seconds before the breaths for V measurements of the V–P point The stage of wakefulness was judged from the EEG pertaining to the period during which the ventilation was measured and to this time the V–P point was referred

c *Calculation of SQ, sT and DsT from the CO tests*

In all the CO tests every other V–P point was calculated For each investigation all these points were plotted in a V–P diagram (cf Fig 19) In this way each test usually consisted of 10 to 15 V–P points

For each individual the scatter of the V–P points representing identical EEG stages of wakefulness was narrow Above a "threshold" value (T) of

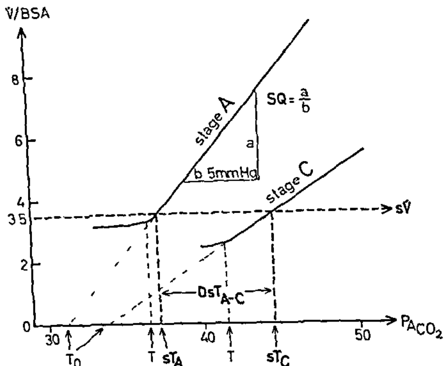


Fig 6 D ag am illustrating main symbols used in the text For explanation see this page and pa e

P_{ACO_2} the curve formed by the V—P points was approximately rectilinear For each stage of wakefulness *the slope* of this line *SQ* (stimulus response quotient or sensitivity quotient) was expressed as V/BSA/P_{ACO_2} in L/min/m²/mm Hg Fig 6 schematically illustrates the main symbols described here

During wakefulness at rest (EEG stage A) and at respiratory steady state the mean V/BSA for all the subjects was approximately 3 5 L/min/m² A line representing this *standard ventilation* sV was drawn in every V—P diagram The P_{ACO_2} value at which a slope line crossed the sV line was termed the *standard threshold* sT for respective stages of wakeful ness Accordingly the difference in sT between two slope lines was named *DsT* (i.e the difference in P_{ACO_2} in mm Hg at the sV) Between EEG stages C and A it was designated as DsT_{A-C} between stages D and A as $DsT_{A\ D}$ and so on

As a rule the slopes of the response curves of each stage of wakefulness in every subject reached the sV line The threshold value T for P_{ACO_2}

Table III *Time delay for a ventilatory response to a transient change in* P_{ACO_2}
(Within brackets the number of determinations)

A		B	
Subject	From end of 40 sec CO_2 rebreathing	Subject	From onset of 2 or 3 single breaths of CO_2 rebreathing
H F	(6) *22 1*	F A	(2) *20 8*
E H	(4) *20 3*	E W	(2) *18 3*
E A	(3) *25 4*	K B B	(2) *27 6*
K B I	(4) *26 0*	A K B	(2) *26 5*
K B II	(4) *26 8*	I Sd	(4) *17 4*
V R	(1) *22 5*	R T	(3) *25 8*
B H III	(3) *20 7*		
K Å N II	(3) *25 9*		mean 22 7
K N I	(3) *22 5*		
A H II	(3) *24 2*		
I S	(2) *26 8*		
S I II	(3) *22 3*		
mean	24 3		

According to the above mentioned time delay, P_{ACO} was estimated from the five breaths occurring approximately 25 seconds before the breaths for V measurements of the V–P point The stage of wakefulness was judged from the EEG pertaining to the period during which the ventilation was measured, and to this time the V–P point was referred

c Calculation of SQ sT and DsT from the CO tests

In all the CO_2 tests every other V–P point was calculated For each investigation all these points were plotted in a V–P diagram (cf Fig 19) In this way each test usually consisted of 10 to 15 V–P points

For each individual the scatter of the V–P points representing identical EEG stages of wakefulness was narrow Above a ' threshold value (T) of

or at the beginning of the test stable respiration (P_{ACO_2} change not exceeding ± 1 mm Hg) and stable FEG for an equally long period (3—4 min)

Marked sudden changes in ventilation occurred in some of the subjects concomitant with rapid changes of wakefulness V—P points falling during such substantial tran ient deviations from a stable re piration were not included in the ventilation CO_2 response measurements

B Controls of ventilatory response to an increase of P_{ACO_2} of different rate

The re ponse was checked when the rebreathing volume in the spirometer system was lower as well as when it was higher than during the ordinary CO_2 test This changed the rate of increase of the CO_2 content of the in piratory air as well as alveolar air The position and slope of the ventilation CO_2 respons curve was studied during stable EEG levels

With a low rebreathing volume of 13 5 L (compared with ordinary 30 L) a moderate shift of the CO_2 re ponse curve to the ri ht was noted in 4 of 6 subjects studied and in 2 of them also a mall decrease in the slope compared with the ordinary CO_2 test.

Twice the normal rebreathing volume 60 L produced no clear difference in po ition or slope of the ventilation CO_2 response curve in 6 subjects studied In 2 of th cases an inconsiderably increa ed slope (SQ) was noted in a few of the 60 L-tests while sT was slightly lower in 3 of the subjects and sli htly higher in 2

The rate of increase of the inspiratory CO_2 tension during these CO_2 rebreathing tests was expressed by the quotient of insp ratory CO_2 tension at the end of the test and the duration of the test. In the 13 5 L-tests (10 tests) the rate varied between 8 and 15 mm Hg/min in the ordinary (30 L) CO_2 tests only between 3 6 and 4 7 mm/min In the 60 L tests (8 te ts) the corre pondin variation was 1 8 to 2 3 mm Hg/min

To conclude during the ordinary CO_2 test (with rebreathing in 30 L volume) there seemed to occur a dynamic" balance between P_{ACO_2} and V not differing clearly from what occurred with half as fast an increase of the inspiratory CO_2 tension in twice the rebreathing volume

C Controls of ventilatory response to increased P_{ACO} values of long duration

In 12 of the examinations different levels of increa ed P_{ACO_2} were maintained for periods of 10 to 20 minutes with CO_2 sh nt tests as described on page 14 This was perfomed at different levels of wakefulness of the subjects For each individual and level of wakefuln s the ventilation CO_2 re ponse was compared with the results from the ord nary CO_2 tests in the V—P diag am

At each level of wakefulness the e was as a rule a slight to moderate sh ft to the left of the V—P po nts from the shunt test" as compared to tho e from the ordinary CO_2 test Furthermore a slight increase in slope SQ was noted The shift in sT as well as in SQ was however approximately the sam at different levels of wakefulness

Figs 23 and 24 (page 59 and 60) illustrate the shift in two typical ca es It is evident that the differences between two EEG stages in sT and in SQ

(above which the ventilation CO_2 response curve was *approximately* linear), usually lay close to the corresponding sT value

The use of $\dot{V}-P$ points suppressed the effects of sudden transient respiratory changes during single breaths on the measured relationship between V and P_{ACO_2}. Even when the P_{ACO_2} could not always be measured from every expiration the mean P_{ACO_2} of the $\dot{V}-P$ point could usually be determined. It was always based on at least 3 end expiratory measurements. Only exceptionally such as during marked hypopnoea was it necessary to apply interpolation

There was also another advantage of the 5 breath system. The duration of 5 breaths, about 25 seconds generally allowed a definite classification of the level of wakefulness from the prevailing pattern in the EEG. During some such periods however the EEG pattern was too unstable to allow classification. This complication occurred especially during transitions between two levels of wakefulness

Besides a correlation to body surface area two other common methods were tried to enable an inter individual comparison of the ventilatory values. The ventilation was expressed either per volume oxygen taken up ($\dot{V}_{O_2}$) or per volume of ventilation during respiration at rest "ventilatory ratio" (dividends in both cases determined at steady state for EEG stage A). Neither alternative proved superior to the body surface method

8 *Evaluation of the respiratory stability*

A *Criteria applied*

During normal night sleep there are frequent, and often quite large spontaneous changes in the depth of sleep (*cf* KLEITMAN 1939 DEMENT & KLEITMAN 1957) as well as in ventilation and in alveolo arterial CO_2 tension (see under Results Chapter III). This fact, as well as the repeated ventilation CO_2 response measurements made it necessary to apply certain criteria on a respiratory "steady state"

During the CO_2 tests the gradually increasing P_{ACO_2} causes a state of respiratory imbalance. However the rate of change of the two main respiratory variables V and P_{ACO} was fairly low and stable even between different individuals. A further decrease in this rate had little influence upon the ventilatory response measured (see further under B below). On the basis of these observations, the following criteria for stable respiration ("steady state") were set up for the present study

1 *Respiration between the CO_2 tests* (*i e* during oxygen enriched air breathing) Stable EEG pattern (level of wakefulness) as well as stable respiration since at least 3 to 5 minutes. The examination conditions completely unchanged for at least 5 to 7 minutes. P_{ACO} varying less than $\pm$ 1 mm Hg

2 *Respiration during CO_2 tests* Stable EEG (level of wakefulness) and respiratory values steadily rising since 3 or 4 minutes (see Fig 3, page 17)

RESULTS

RESPIRATION AND WAKEFULNESS DURING AIR BREATHING

A *Respiration at different EEG stages of wakefulness*

General findings — Changes in EEG between wakefulness drowsiness and sleep were invariably accompanied by changes in ventilation as well as in alveolar carbon dioxid tension The respiratory changes during transition between wakefulness drowsiness and sleep were recorded on more than 1 000 occasions

A fall in wakefulness from stage A to stage B or from B to C or further to still deeper sleep was regularly accompanied by a decrease in ventilation and an increase in $P_{A_{CO_2}}$ A rise in wakefulness on the other hand was accompanied by an increase in ventilation and a reduction in $P_{A_{CO_2}}$ These respiratory changes also occurred during abrupt transient shifts in wakefulness as judged from the EEG They appeared even during only a few seconds or less during parts of an expiration or an inspiration In contrast to the change in ventilation the change in $P_{A_{CO_2}}$ was always more gradual during the course of some breaths

Figs 7 and 8 show typical changes in ventilation (V) and $P_{A_{CO_2}}$ between consecutive breaths during a period in which the EEG pattern fluctuated between various levels of wakefulness When the alpha activity in the EEG disappeared and theta waves developed ventilation decreased When the alpha activity reappeared sporadically ventilation again increased The accompanying change in $P_{A_{CO_2}}$ was more successive

Figs 9 and 10 show the changes when the subjects awoke from deep sleep With the occurrence of more rapid frequencies in the EEG which became dominated more and more by alpha activity the ventilation increased abruptly The following decrease in $P_{A_{CO_2}}$ occurred more slowly After a few breaths the initial marked increase in ventilation showed a slight reduction The respiration then gradually became stable around values normal for wakefulness

Figs 8 and 9 show another characteristic difference between the changes noted when the subject was falling asleep and when he was waking up The transition from wakefulness stage A to uninterrupted sleep (stage C or lower) was never abrupt When the subject was *falling asleep* there was a long period of fluctuating wakefulness with repeated periods of signs

were not substantially altered during the respiratory stabilization during the "CO_2 shunt tests"

Standard errors of the main values measured are given (within parenthesi) together with the corresponding mean values A convenient formula was used

For obvious reasons a study of the pre ent type could not be scheduled beforehand with stringent demands on statistical trials of significances Stable sleep of various depth and duration cannot be induced at will Reinvestigations were made *in* several but — *for practical reasons — not in all subjects In ome cases several reinvestigations* were made

In the calculation of standard error the mean values *for all* investigations of each subject were used It should therefore be stressed that the errors here given are in reality smaller since the increased accuracy with use of the mean values of repeated investigations in each subject is not considered in the calculations

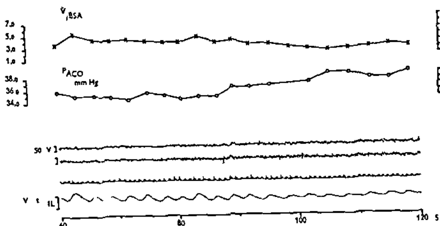


Fig 8 Subj B H male a ed "3 A phase of gradual decrease in wakefulness during the period of onset of sleep Concomitant changes in respiration.

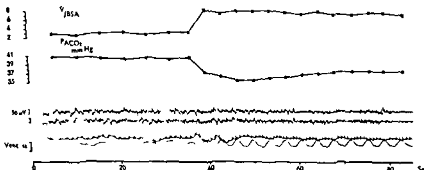


Fig 9 Subj F A male aged 39 Typical (pontaneous) *arousal from deep stable sleep* Not the sudden increase in ventilation (see also the spirographic record lowermost) and the somewhat slower ch n_e in P_{ACO_2} A gradual stabilization in respiration follows (The first signs of alpha act vity in the EEG u ually preceded the change in ventilation by one or two seconds during awakenin_)

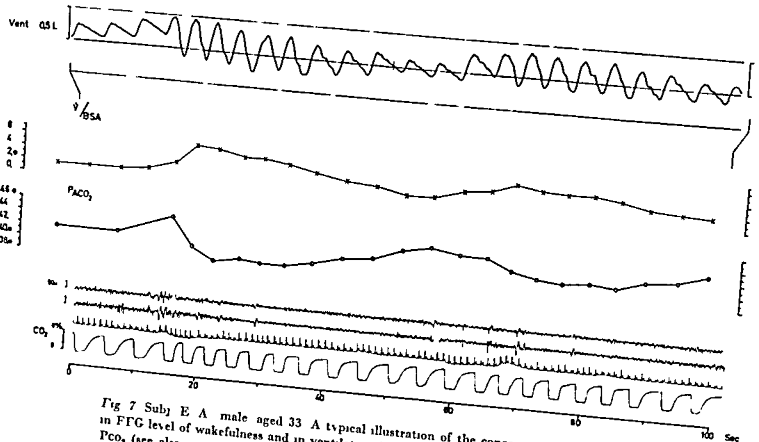
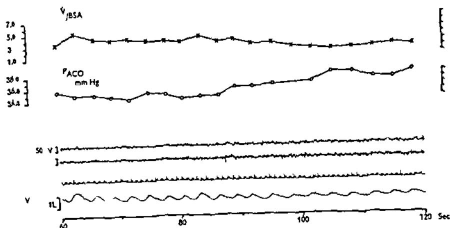
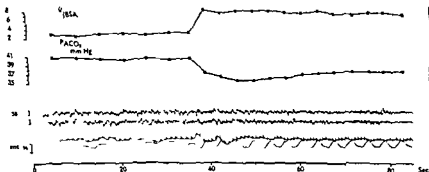


Fig 7 Subj E A male aged 33 A typical illustration of the concomitant variations in EEG level of wakefulness and in ventilation (spirographic record above) and alveolar P_{CO_2} (see also capnographic record lowermost) during the *period of onset of sleep* The *periodic breathing* (PB) is here of moderate degree



F g 8 Subj B H male a ed 23 A phase of gradual decrease in wakefulness during the period of onset of sleep Concomitant changes in re piration



F g 9 Subj F A male a ed 39 Typical (pontaneous) *arousal from deep stable sleep* Not the sud len increase in ventilation (ee also the spirographic record lowermost) and the somewhat slower chan e in P_{ACO_2} A gradual stabilization in respiration follows (The first s gn of alpha acti ity in the EEG u ually *preceded* the change in ventilation by one or two seconds during awakening)

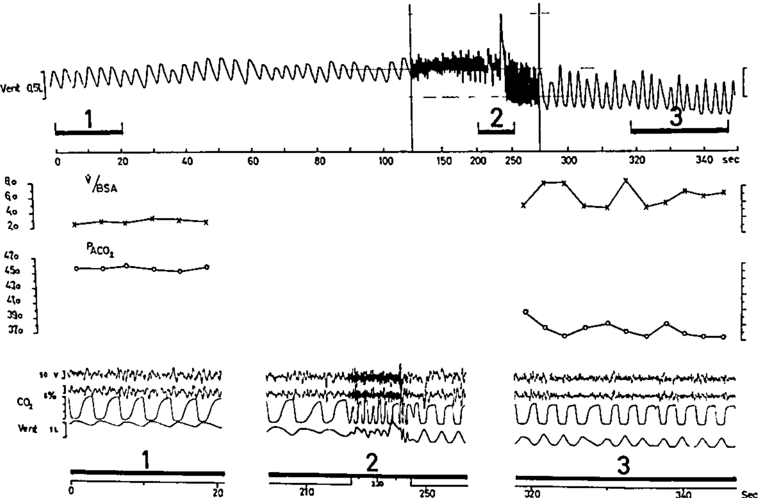


Fig 10 Subj K N male aged 27 Typical respiratory pattern during a period of about 6 min showing deep sleep heavy arousal reaction and ensuing wakefulness Three sections of the spirographic record (above) are also illustrated by simultaneous EEG and capnographic records (on a larger time scale below)

of drowsiness or sleep in the EEG which were accompanied by a decrease in ventilation and an increase in P_{ACO_2}. These periods were interrupted by periods of higher level of wakefulness characterized by alpha activity and an increase in ventilation.

The process of *awakening* was always shorter than that of falling asleep. Already within the first or second breath during an arousal reaction with occurrence of alpha waves in the EEG the marked increase in ventilation was usually maximal (Fig. 9). In contrast to what was seen when the subject was falling asleep prominent undulations in level of wakefulness were seldom observed following arousal. Both EEG and respiration became stable more rapidly. When the subject awoke only for a short time a new unstable period followed with the typical variations in wakefulness and respiration. Very transient arousal reactions were usually followed more quickly by definite sleep.

Fig. 9 also illustrates that during arousal from deep sleep the first sign of alpha activity often preceeded the increase in ventilation by 1 to 2 sec.

During shifts from light to moderately deep sleep the *same* principal changes in respiration as described were also *observed*. They were invariably present in association with the occurrence of K complexes in the EEG. During deep sleep (stage D and E) respiration was *in general more* stable. Small or slow changes in the pattern of deep sleep were *not* always accompanied by demonstrable respiratory changes.

Respiration and level of wakefulness during "steady state." Fig. 11 represents *one of* the principal findings in the subjects studied. When the EEG showed a stable pattern at lower levels of wakefulness respiration was also stable with only slight *breath to breath* variation in V/BSA ± 0.5 L/m² and P_{ACO_2} ± 0.5 mm Hg indicating the occurrence of a new respiratory equilibrium between the low ventilation and the high P_{ACO_2}. In prolonged sleep of constant depth the ventilation was always very uniform from breath to breath.

This figure shows the consecutive breath by breath variations *in* respiration in a V-P diagram for one subject. The diagram covers 3 *periods* of different level of wakefulness (stages A awake, B drowsy and D, deep sleep). During all 3 stages the EEG pattern was stable. The simultaneously measured V-P values of each single breath during sleep are clearly separated from those during wakefulness, the former being situated in the lower right part of the diagram. Stage B is *an* intermediate stage in the EEG and, as is clear from the diagram, also in the *respiration*. *In all* 3 stages the variations between consecutive breaths are very small in ventilation and P_{ACO_2}.

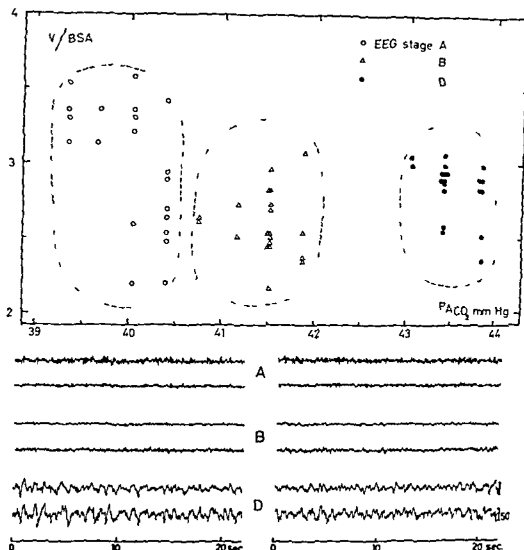


Fig 11 Subj B B male aged 23 *Breath by breath variations in ventilation and* P_{ACO_2} during three separate periods (each of 77 seconds duration) representing *stages A (awake) B (drowsy) and D (deep sleep)* respectively The EEG records below refer to the first and the last 22 seconds of each period Note the clear separation in the diagram of points belonging to different stages of wakefulness each stage with a stable EEG pattern Points representing sleep are all situated below to the right as compared to during wakefulness (stage A) the points during drowsiness situated in between Drowsiness is however often less stable in EEG as well as in respirat on

(In this diagram V and P_{ACO_2} *of each breath repre ent simultaneous measurements* whithout the time delay used for V P points)

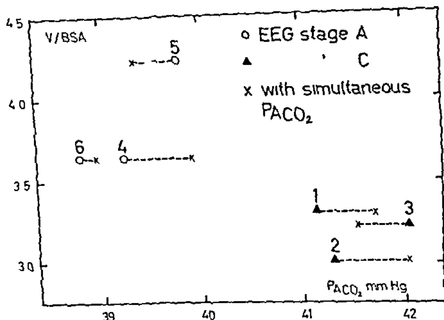


Fig 12 *A* Subj T S male aged 18 V P points from wakefulness and sleep The points are numbered chronologically (Between point 3 asleep and point 4 awake the time interval is more than 2 hours) The broken lines denote the differences in P_{ACO_2} between measurements made simultaneously and at the routine interval of 25 sec in relation to the ventilation measured The small differences indicate a stable respiration
Note that points during stable sleep are situated below to the right of points during wakefulness stage *A*

Similar stable conditions in respiration and in EEG pattern are illustrated in Fig 12 A and B Each diagram shows V P points from widely different parts of a 5 hour recording period of one investigation and during different levels of wakefulness For each stage of sleep (stages C and D respectively) the plottings lie close together The spread is somewhat larger for stage A

The size of change in respiration between wakefulness and sleep was usually of similar order in the same individual This too is demonstrated in Fig 12

Sometimes as is shown in Fig 13 some shift was observed in the position of the V P points in the diagram — from wakefulness as well as sleep — between various sections of a record traced for several hours The principal change in position of the V P points between two levels of wakefulness was however always distinct for each such part of a given recording

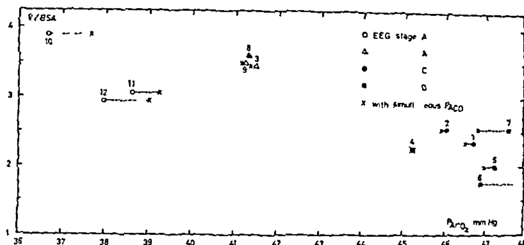


Fig 12 B K A N male aged 27 V P points from four separate stages of wakefulness (including tense alertness stage A_a) during a 5 hour recording period The points are *numbered chronologically As in Fig 12 A the* P_{ACO_2} *values measured simultaneously with the ventilation are also included* in order to illustrate the degree of respiratory stability at each V P point (Since the two P_{ACO_2} values coincide in point 8 stage A the open triangle is indistinct in the reproduction)
Note that with a decrease in wakefulness the points are shifted downwards to the right

It should be stressed that changes in position of the plottings for identical EEG stages between different periods of a given investigation can not be ascribed to technical errors of the CO_2 analyzer which had very small drift and was repeatedly checked during the recording periods by gas calibrations (page 15) It seems however possible that these changes in position of V P points between different parts of one recording period are largely due to protracted differences in the CO_2 output (see below page 43)

During longer periods of stable wakefulness or sleep as reflected in the EEG V P points were determined at the beginning middle and end of the period Fig 13 shows that in each group the corresponding points in the V P diagram coincided well indicating stable respiration

When profound and stable sleep had lasted for a long time a slight diminution of the change in respiration was sometimes observed The carbon dioxide output and the respiratory exchange ratio had then reincreased indicating more stable respiratory conditions The principal difference in position in the V P diagram compared with that of wakefulness however always persisted

Measurements of the quantitative changes in the respiratory variables between wakefulness and sleep will be reported in detail in chapters V and VI The average ventilation per m^2 body surface area decreased by 0 9 ($\pm$ 0 1) L/min , the P_{ACO_2} increased by 3 1 ($\pm$ 0 3) mm Hg between the EEG stages A and C in an analysis of 29 subjects all of whom showed periods of stable sleep (*see also Table VIII, page 67*)

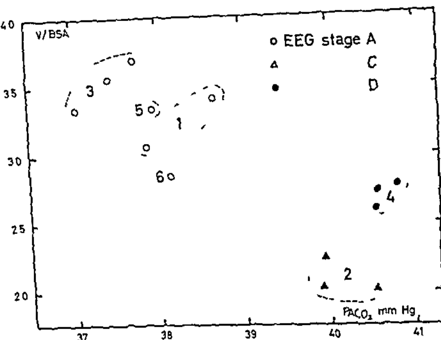


Fig 13 Subj I A female aged 2? V P points from widely separated periods of stable EEG le el of wakefulness during a 5 hour recording period For each period the points are circumscribed by a broken line In group 4 (deep sle p) there was a well developed respiratory steady state apparently more marked than in group 2 (light sleep) The groups are numbered chronologically S e text

The quotient between V/BSA and P_{ACO_2} decreased gradually between stages A B and C Taking the stage A quotient as unity the mean value for stage C in the e subjects was 0 70 (± 0 02)

Pattern of ventilation during sleep During sleep the type of ventilation as judged from the individual breaths on the *spirogram* was found to be characteristically changed When the EEG showed a stable pattern the breaths showed only small variations in volume and frequency In addition the interindividual variations in ventilatory frequency proved to be reduced (Table VII column 5 page 66) Consequently during sleep the differences in ventilation between the subjects were mainly reflected in the tidal volume

The ratio between the duration of inspiration and of expiration was usually decrea ed during sleep most markedly at the onset of drowsiness or sleep This implied that the average volume expired per unit time was

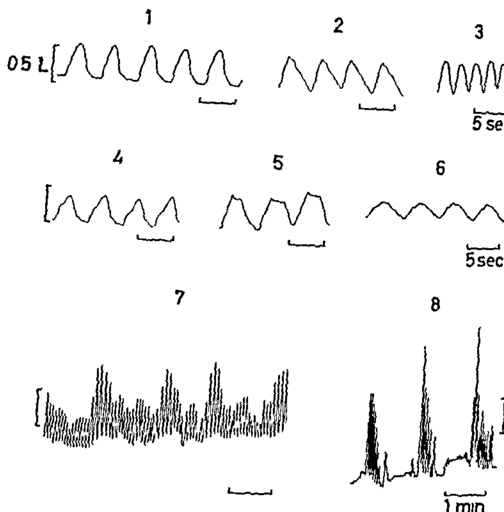


Fig 14 Pattern of ventilation during sleep 1 Typical spirographical tracing during wakefulness (stage A) *There is often a distinct expiratory pau e* 2 *The most common pattern during light and moderately deep sleep There are slight undulations already early during* expiration 3 One variant (stage C) 4 Less common form moderately deep leep The ratio inspiration time/expiration time is here increa ed (not decrea ed as in 2) 5 and 6 Deep sleep (stages D or E) 7 Typical periodic breathing (PB) of slight to moderate degree concomitant to variations in wakefulness 8 Marked PB with phases of apnoea or marked hypopnoea In each period the ventilatory maximum is reached during the first one or two breaths following an arou al

decreased In some individuals the ratio was however rever ed when the EEG showed deep sleep In a few individuals such an increase of the ratio occurred already at the onset of sleep During deep sleep of long duration (30 min or more) the difference in this ratio compared with that in wakefulness was often less marked

A further characteristic phenomenon was the disappearance of the end

expiratory pause at onset of sleep The transition between inspiration and expiration was also more sharply defined The curve during expiration showed a smoother gradient indicating that the maximal (resting) expiratory flow rate was reduced There was usually a slight undulation at the middle of the expiration Typical sleep breaths are illustrated in Fig 14 compared with typical breaths during wakefulness In some individuals there were transitory changes of the end tidal level (usually a rise in some subjects alternating with a decrease) when wakefulness decreased rapidly (Figs 7 and 14 7 8)

Since the characteristic changes in the pattern as well as in the degree of ventilation were most pronounced at the onset of sleep *it was always possible to recognize even very transient periods of sleep from the spirographic record alone*

During deep sleep expiration sometimes showed a marked dissociation in time between an initial movement of the abdominal wall (diaphragm) and a contraction of the chest first later in the expiration The initial phase produced only a slight change in volume in the spirometer and was followed by a distinct pause (Fig 14 5)

Snoring occurred at different sleep levels It was often associated with EEG changes indicating a fluctuating wakefulness

Also during deep sleep with a stable EEG irregular changes occurred in the shape of separate snoring breath This occasionally produced transient changes in the degree of ventilation and in P_{ACO_2} but the respiration remained stable in between

B *Respiration during tense alertness*

During a state of tense alertness a slight mean increase occurred in ventilation and also a slight decrease in alveolar carbon dioxide tension

A stable state of alertness was difficult to maintain. During repeated attempts to hold the alert level for several minutes the respiratory variations were sometimes appreciable It was therefore often not possible to secure respiratory stability at fying the criteria otherwise applied (page 28) The studies of alertness are reported further in Chapter V (page 57)

C *Variations in respiration during a complete period of recording*

Fig 15 shows how V P point values for P_{ACO_2} and V varied throughout the examination period in 1 subject These V P points were determined from respiration in (oxygenated) air at 5 minute intervals (All values which fell within 5 minutes after a CO_2 test or within 3 minutes after a transient change in the composition of the inspiratory gas such as after determination of P_{VCO_2} were excluded) Since the V P points were thus selected irrespective of the degree of stability of the EEG level of wakefulness i e including also points during transitions between the EEG stages the spread at

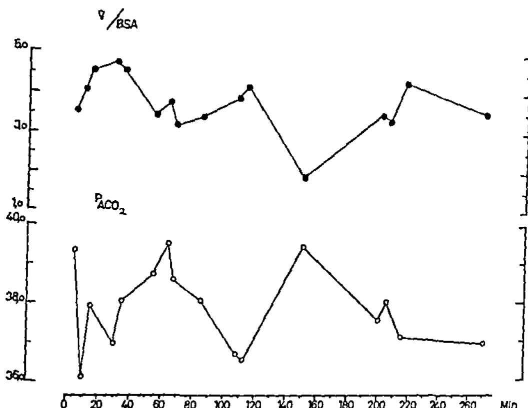


Fig 15 Subj E N male aged 39 Variations in ventilation and P_{ACO_2} from V P points measured at 5 minute intervals during one recording period (For values excluded see text) The points at the beginning around 60 and at 150 minutes represent sleep periods At 10 around 110 and after 210 minutes the subject was awake

different levels of wakefulness is here fairly wide It was however clear that the variation in respiration largely followed the EEG level of wakefulness During wakefulness stage A the ventilation is high the P_{ACO} low, during sleep the reverse is noted

Fig 16 shows the V and P_{ACO_2} distribution among the V P points (selected in the way just described) for 4 other subjects who, like the case in Fig 15 slept for substantial parts of the recording period Each solid square corresponds to V and P_{ACO} , respectively of a V P point The spread of V and P_{ACO_2} which differs from person to person, is commented upon in Chapter VI

D *Carbon dioxide output oxygen uptake, alveolar ventilation and respiratory dead space at different levels of wakefulness*

V_{ECO_2} The CO_2 output at different levels of wakefulness is summarized in Table IV During the early phases of the onset of sleep with a particularly low ventilation, the V_{ECO_2} was also at its lowest

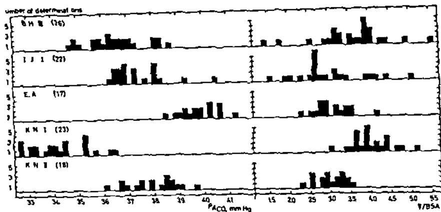


Fig 16 D stribut on of values for ventilation and P_{ACO_2} from V P points measured at 5 minute intervals (see t xt) in 5 investigations (4 subjects) The number of V P points in each investigation is given in brackets Each recording period lasted 4 to 5 hours and conta ned comparable lengths of sleep

The scatter is somewhat wider in the two uppermost cases They belonged to the "un stable" group (page 62) while the remain ng two ca es were stable

In some subjects this occurred already during li ht drowsiness (stage B_1) when there were sporadic but still distinct short alpha periods in the EEG In more cases however the $\dot{V}_{ECO_2}$ was lowest during clear cut stage B with a dom nating low voltage theta and no alpha activity in others the decrease in $\dot{V}$ and $\dot{V}_{ECO_2}$ was greatest during the initial period of very light sleep corresponding to stage C or a transitional stage between B and C.

With the stabilization of respiration during uninterrupted sleep the $\dot{V}_{ECO_2}$ again increa ed and approached that of stable wakefulness

$\dot{V}_{O_2}$ It was observed (Table IV) that the oxygen uptake changed less than the carbon dioxide output during the onset of sleep as well as between different more stable stages of wakefulness In some cases the $\dot{V}_{O_2}$ was measured repeatedly during periods of continuous sleep The values were then somewhat lower towards the end of the period

R. It is clear from the fo egoing that the quotient between CO_2 output and O_2 uptake R always decreased when the subject was falling asleep to ris again gradually as re piration became more stable during uninterrupted sleep With the establishment of a new re p ratory equilibrium, the respiratory quotient approached the same value as during wakefulness

$\dot{V}_A$ and $\dot{V}_D$ The alveolar ventilation $\dot{V}_A$ was found to be lowered during sleep (Table IV) The reduction was roughly proportional to the decrease in ventilation and in tidal volume The calculated mean decrease in respiratory dead space was only small and not a certained

Table IV CO_2 *output* O_2 *uptake dead space volume alveolar ventilation and alveolar/total ventilation ratio in subjects awake drowsy and asleep*

Subject	V_{ECO_2} ml/min			V_{O_2} ml/min			V_D ml			$\dot{V}_A$ L/min			V_A/V		
	stage A	stage B	stage C	stage A	stage B	stage C	stage A	stage B	stage C	stage A	stage B	stage C	stage A	stage B	stage C
A A	190	160	140	240	220	220	150	100	120	3 6	3 2	2 7	0 64	0 66	0 60
I B A	220	190	190	240	250	240	140	110	120	4 4	3 5	3 6	0 65	0 64	0 59
C H	170	180		230	230		120	120		3 1	3 3		0 64	0 70	
F A	200	170		270	280		110	110		3 6	3 0		0 73	0 65	
S F	200	240		300	340		140	120		3 9	4 7		0 66	0 71	
F N I	200		180	230		240	130		140	3 8		3 3	0 63		0 77
L K	240	170	240	290	260	290	130	120	110	4 5	3 3	4 5	0 71	0 66	0 72
L H	280	250		310	330		150	150		5 1	4 6		0 60	0 57	
R T	210	150		280	290		130	140		4 1	3 1		0 66	0 62	
V R	190	190		210	210		160	150		3 7	3 5		0 60	0 60	
I A I	190		160	200		220	100		120	3 6		2 7	0 73		0 63
I J I	1,0	170		210	210		120	120		3 4	3 3		0 62	0 65	
K N H		150	180		200	200		100	90		3 0	3 5		0 58	0 64
K A N H	310	150	170	290	250	250	160	120	130	6 1	2 5	3 0	0 69	0 64	0 68
mean	*220*	*180*	*180*	*250*	*260*	*240*	*130*	*120*	*120*	*4 0*	*3 4*	*3 3*	*0 66*	*0 64*	*0 66*

The mean quotient $\dot{V}_D/\dot{V}_I$ was 0 34 ($\pm$ 0 01) during wakefulne s and 0 34 ($\pm$ 0 02) during sleep Thus the quotient did not change noticeably between wakefulness and sleep and there was only moderate scatter between the ubjects

As shown in the previous chapter (pa e 23) the P_{ACO_2} determined by capnography was on the avera e 3 mm lower than the P_{CO_2} as measured in arterial blood This fact should be taken into account when comparing the values given for both $\dot{V}_A$ $\dot{V}_D$ and the quotient $V_D/\dot{V}_I$ for these values were calculated with the aid of the capnographically determined P_{ACO_2} (see formula on page 17)

RESPIRATORY VARIATIONS DURING ONSET OF SLEEP

A *Onset of sleep and periodic breathing*

The process of falling asleep was invariably accompanied by repeated periods of decrease in the EEG level of wakefulness In more than two thirds of the subjects there was during the periods of onset of sleep periodic breathing (PB) of a more or less pronounced degree, implying undulations in ventilation (V) and P_{ACO_2} as measured breath by breath This type of breathing (PB) (Figs 3, 7 and 14) often lasted for 10—20 minutes some times even longer The "wave length" of each breathing period for V as well as for P_{ACO_2} was usually between 60 and 75 seconds It rarely fell below 40 seconds, and then only slightly

As mentioned in the previous chapter a decrease in wakefulness was invariably accompanied by a decrease in ventilation The latter decrease was rapid and often became maximal within the first few breaths some times even developed into transient apnoea The more rapid and marked the initial fall in level of wakefulness, the more pronounced was the reduction in ventilation and the ensuing increase in P_{ACO_2} as well as the degree of PB

The period of onset of sleep Onset of sleep was accompanied by periods of irregular, frequent variations, as well as of regular undulations in wake fulness and in respiration

As a rule onset of sleep was initiated by a period of frequent variations in both wakefulness and respiration This *introductory phase* was followed by an *intermediate phase* with reduced frequency of wakefulness changes in the EEG and a more regular periodicity of breathing and of wakefulness There occurred a further decrease in wakefulness and increase in mean P_{ACO_2} Usually a *final phase* could be recognized, during which definite sleep occurred There was then a gradual decrease in the PB while the P_{ACO_2} became stable at a higher level

In several subjects the ventilatory changes during periodic breathing were so marked and regular that they closely resembled CHEYNE STOKES *breathing* As a rule however the changes in ventilation were more abrupt than in CHEYNE STOKES' breathing, especially in the presence of conco

mitant periodic arousal reactions in the EEG (Fig 14 B) In some cases periods of total apnoea for 10 to 20 seconds or more occurred occasionally for more than 1 minute Usually however, there was hypoventilation instead of apnoea

Marked irregular changes in ventilatory pattern (even resembling BIOT s breathing) were noted in some subjects during the introductory phase of the onset of sleep concomitant to variations in the EEG pattern of wakefulness Some characteristic respiratory patterns during onset of sleep are shown in Figs 7 8 and 14 A B

B *Observations on P_{ACO_2} at low levels of wakefulness*

Apnoea or pronounced hypopnoea occurred only in the introductory phase of the onset of sleep and then only when the preceding P_{ACO_2} was relatively low situated in the V P diagram far to the left of the rectilinear part of the ventilation CO_2 response curve for the lower stage of wakefulness during the hypopnoic phase When during a phase of apnoea or marked hypoventilation the successively increasing P_{CO_2} reached values around the CO_2 threshold (T) of the response curve the ventilation usually started or increased anew It was then often followed by elevation of the level of wakefulness In some cases however the threshold was first markedly exceeded and then a sudden ventilatory increase appeared concomitant with an arousal reaction in the EEG

CO_2 arousal threshold In 13 subjects all with obvious periodicity in respiration and wakefulness while slowly falling asleep the P_{ACO_2} values were measured immediately preceding or (when not measurable then) at the very beginning of arousal reactions in the EEG These P_{CO_2} values were noted from periods during breathing (oxygen enriched) air as well as during CO_2 rebreathing Apparently spontaneous awakenings from sound sleep were not included

All the P_{ACO_2} values were grouped according to the level of wakefulness just before the arousal For every recording period and level of wakefulness the variation in these P_{ACO_2} values was small the range usually less than $\pm$ 1 mm Hg The lower the level of wakefulness the higher was the P_{ACO_2} at the time of the arousal Table V summarizes these results

Comment The comparatively small variation in these CO_2 values of an individual at each wakefulness level may suggest a causal relationship between the high CO_2 tension and the occurrence of the arousal On this account these P_{ACO_2} values were regarded as ' CO_2 arousal thresholds ' (see further under Discussion)

CHAPTER IV

RESPIRATORY VARIATIONS DURING ONSET OF SLEEP

A *Onset of sleep and periodic breathing*

The process of falling asleep was invariably accompanied by repeated periods of decrease in the EEG level of wakefulness In more than two thirds of the subjects there was during the periods of onset of sleep periodic breathing (PB) of a more or less pronounced degree, implying undulations in ventilation (V) and P_{ACO_2}, as measured breath by breath This type of breathing (PB) (Figs 3 7 and 14) often lasted for 10—20 minutes some times even longer The "wave length" of each breathing period for V as well as for P_{ACO_2} was usually between 60 and 75 seconds It rarely fell below 40 seconds and then only slightly

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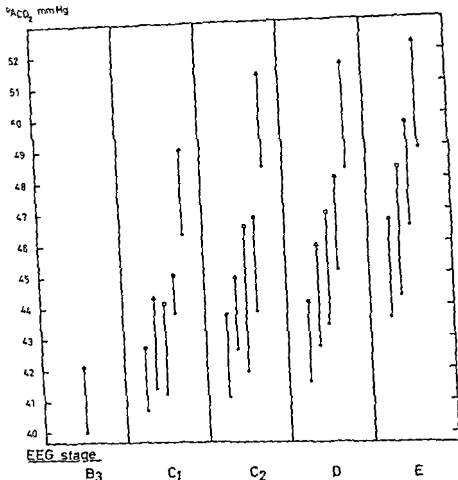


Fig 17 Range of variation in P_{ACO_2} in five subjects (K. N female aged ?1 S I male aged ?1 B B male aged 3 K Å N male aged 27 and K. B female aged 42) *for stages of sleep* (B_3 denotes very light sleep and stage C is divided into two subgroups C_1 and C_2 indicating slightly increasing depths of sleep) Upper value in each case and stage mark the "CO_2 *arousal threshold*" (see text)

shifts to lightly lower EEG levels of wakefulness (here called stages B_3 C_1 C_2) implied some increase of the P_{ACO_2} values as is apparent from the figure

CO_2 rebreathing during onset of sleep and during continuous sleep The successive increase in P_{ACO_2} during the *CO_2 tests* included a gradu

Table V CO_2 *arousal thresholds at different EEG stages of sleep*
(Within brackets the number of determinations)

B_3 denotes very light sleep Stage C is divided into two subgroups C_1 and C_2 indicating slightly increasing depths of sleep

Subj	stage B_3	stage C_1	stage C_2	stage D	stage E
M L		(5) *45 4*	(30) *46 9*	(9) *48 6*	
F A	(2) *42 2*	(9) *43 0*	(5) *44 9*	(2) *46 6*	
K Å N I		(2) *45 0*	(4) *46 8*	(5) *48 0*	([illegible]) *49 7*
B B II		(6) *44 1*	(13) *46 5*	(3) *46 9*	(2) *48 3*
M W		(2) *43 7*	(14) *44 1*	(3) *45 0*	(1) *49 0*
B H III	(1) *39 8*	(8) *40 8*	(14) *41 9*	(3) *42 4*	(2) *44 1*
K B II		(9) *49 0*	(25) *51 3*	(7) *51 6*	(2) *52 2*
A H I	(4) *36 5*	(11) *37 7*	(3) *38 8*	(2) *40 8*	
S I I		(5) *44 3*	(7) *44 9*	(3) *45 9*	(11) *46 7*
I A II	(1) *39 5*	(19) *40 1*	(6) *40 3*	(4) *41 9*	
I J II		(10) *40 5*	(17) *41 4*	(4) *42 1*	(1) *4[illegible] 0*
E N II	(26) *42 1*	(53) *43 3*	(19) *44 0*		
K N III	(6) *42 1*	(9) *42 7*	(6) *43 7*	(3) *44 1*	

The range of variability in P_{ACO_2} for each stage of sleep was narrow and the limits were fairly stable during one and the same recording period. In deep sleep (stages D and E) the P_{ACO_2} sometimes exceeded 50 mm Hg before an arousal reaction or a change in that direction appeared in the EEG, accompanied by a marked increase in ventilation. On reinvestigation of the same subject the P_{ACO_2} range for each stage of sleep sometimes shifted somewhat, always however in the same direction and to the same degree.

Fig 17 shows the CO_2 ranges of 5 representative subjects. For obvious reasons the arousal reactions observed from deep sleep were few in each investigation. However, within the limits of the EEG stages B to C even

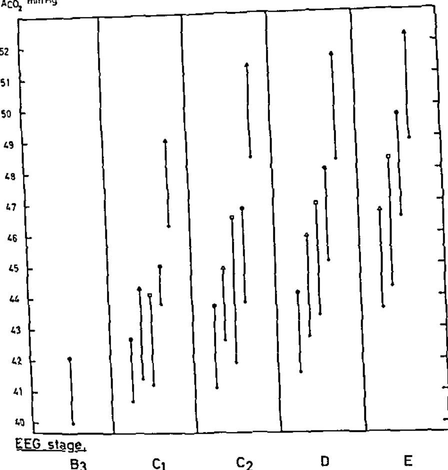


Fig 17 *Range of variation in* P_{ACO_2} in five subjects (K N female aged 21 S I male aged 21 B B male aged 23 K Å N male aged 27 and K B female aged 4?) *for five stages of sleep* (B_3 denotes very light sleep and stage C is divided into two subgroups C_1 and C_2 indicating slightly increasing depths of sleep) Upper value in each case and stage marks the "CO_2 *arousal threshold*" (see text)

shifts to slightly lower EEG levels of wakefulness (here called stages B_3 C_1 C_2) implied some increase of the P_{ACO} values as is apparent from the figure

CO_2 rebreathing during onset of sleep and during continuous sleep The successive increase in P_{ACO_2} during the *CO_2 tests* included a gradual decrease of PB The wave length of the respiratory undulations however

showed no definite tendency to change Somewhat later the concomitant EEG variations in wakefulness showed a tendency to disappear, too

A drowsy subject often fell asleep very easily during a CO test This phenomenon appeared to be due to the fact that as, during the CO_2 rebreathing, the respiratory periodicity became less accentuated, the increased P_{ACO_2} became more rapidly stable within the above mentioned P_{CO_2} range for the sleep stage If, during the further course of the CO test the P_{ACO_2} exceeded the "arousal threshold", the subject woke up and remained awake during the rest of the test When, however, the depth of sleep increased further during the test, sleep often continued through and after termination of the test During the following rapid fall in inspiratory P_{CO} the ventilation then gradually decreased and became stable without any prominent undulations If, however, during a CO_2 test, the P_{ACO} exceeded about 50 mm Hg the subject always woke up even if he was in a deep sleep

At other times, particularly when the subject was in the initial phase of sleep onset, he became clearly awake when the PB variations ceased during the CO_2 test This often followed upon a strong arousal reaction, preceded by a phase of hypoventilation and high P_{ACO} In these cases a distinct PB usually reappeared one or a few minutes after discontinuation of the test There were then typical fluctuations in the EEG level of wakefulness, too

CHAPTER V

VENTILATORY RESPONSE TO CARBON DIOXIDE ("CARBON DIOXIDE SENSITIVITY") AND WAKEFULNESS

The response of ventilation to gradually augmented P_{ACO_2} during different EEG levels of wakefulness was studied in more than 300 CO_2 tests. As described on page 26, the results of the tests were plotted in V P diagrams as response curves.

In many cases the slope of the CO_2 response curve approached the horizontal level at low P_{ACO_2} values (page 26). This was the fact irrespective of the prevailing level of wakefulness. Above a limited range of P_{ACO} values, however, there was a distinct slope (SQ) and this part of the curve was roughly rectilinear (Figs 6-18). This was especially obvious in situations with regular, even, undisturbed breathing and a stable EEG pattern.

During every recording period the position as well as the slope of the rectilinear part of the response curve in the V P diagram was largely unchanged for identical levels of wakefulness. It was also found that when the investigation was repeated in the same subject after an interval of months or even 1—3 years, the slope of the response curve remained strikingly constant. The position, however, of the response curve in the V P diagram could have changed, but always in the same direction and to about the same degree for all stages of wakefulness (Fig 29).

The slope as well as the position of the ventilation CO_2 response curves for identical levels of wakefulness varied widely from one individual to another. These quantitative interindividual differences are evaluated in the next chapter.

A *Threshold and slope of the CO response curve during wakefulness at rest (EEG stage A)*

As described on page 27, the position of a CO_2 response curve in the V P diagram was defined by the CO_2 standard threshold, sT, and the slope of the rectilinear part of the response curve by the stimulus response quotient, SQ. The mean SQ of all stage A response curves was 0.8 ($\pm$ 0.0) L/min/m²/mm Hg, the mean sT_A was 38.6 ($\pm$ 0.4) mm Hg.

The interrelationship of position and slope of the response curves was studied for all the subjects during wakefulness (stage A). When the subjects

showed no definite tendency to change. Somewhat later the concomitant EEG variations in wakefulness showed a tendency to disappear, too.

A drowsy subject often fell asleep very easily during a CO_2 test. This phenomenon appeared to be due to the fact that as during the CO_2 rebreathing, the respiratory periodicity became less accentuated, the increased P_{ACO_2} became more rapidly stable within the above mentioned P_{CO_2} range for the sleep stage. If, during the further course of the CO_2 test the P_{ACO_2} exceeded the "arousal threshold", the subject woke up and remained awake during the rest of the test. When, however, the depth of sleep increased further during the test, sleep often continued through and after termination of the test. During the following rapid fall in inspiratory P_{CO_2} the ventilation then gradually decreased and became stable without any prominent undulations. If, however, during a CO_2 test, the P_{ACO_2} exceeded about 50 mm Hg, the subject always woke up even if he was in a deep sleep.

At other times, particularly when the subject was in the initial phase of sleep onset, he became clearly awake when the PB variations ceased during the CO_2 test. This often followed upon a strong arousal reaction preceded by a phase of hypoventilation and high P_{ACO_2}. In these cases a distinct PB usually reappeared one or a few minutes after discontinuation of the test. There were then typical fluctuations in the EEG level of wakefulness, too.

sT_A. The scatter of these two values between the individuals was appreciable in both groups and there was a wide overlapping in sT_A.

B. *Ventilatory response to CO_2 during lower levels of wakefulness*

A fall in wakefulness was invariably accompanied by a downward shift to the right in the position of the CO_2 response curve in the V-P diagram. This implies that the ventilation for a given P_{ACO_2} value was always lower at a lower level of wakefulness.

Any arousal reaction or change in that direction was immediately followed by an increase of ventilation/P_{CO_2} response and then the response curve shifted upwards and to the left in the V/P diagram. The more marked the change in the EEG, the greater was this upward shift of the curve to the left. It was therefore especially pronounced following arousal from deep sleep.

During comparable states of respiratory stability the largest shift in position (sT) of the CO_2 response curve was in general found between stage A (awake) and stage C (light sleep). As a rule, however, the shift was evident already between stage A and stage B (drowsiness).

For 2 of the subjects the V-P points of CO_2 tests during different EEG stages are shown in Figs 18 and 19. The shift of the ventilatory response to CO_2 with the EEG stage is obvious. (For clarity only two CO_2 response lines have been drawn for each individual.)

Between light and deep sleep (EEG stages C and D or E, respectively) the shift downwards to the right of the ventilation/CO_2 response was as a rule obvious too. During deep sleep (stages D and E) respiration was often very stable. As mentioned in Chapter III, slow transitions between these two stages were sometimes not demonstrable in the respiration. The difference in sT between these two stages, DsT_{D-E}, was usually small and sometimes uncertain.

It is clear from the foregoing that the close interrelationship existing between the shift of the response curve and the corresponding change in the EEG stage of wakefulness does not imply also a quantitative correlation between the DsT value and the shift from one EEG stage to another. Between e.g. stages C and E the DsT was usually smaller than between stages A and C.

In several subjects there was a slight to moderate decrease of the slope SQ of the ventilation/CO_2 response curve during sleep. For several other cases, however, a decrease in slope was not very clear. In no case was an increase in SQ noted during sleep.

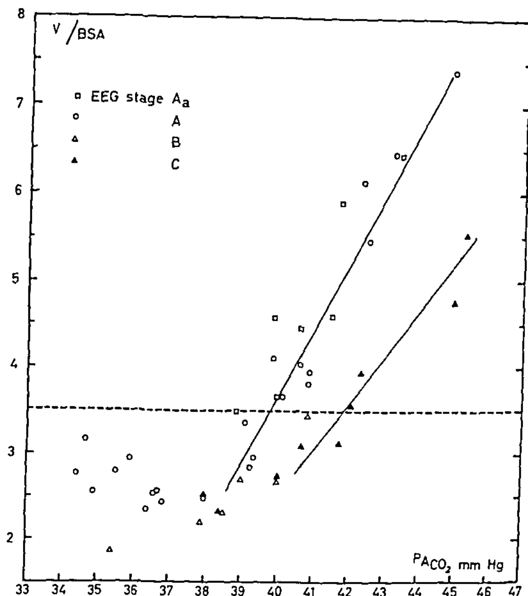


Fig 18 A O female aged 26 Ventilation CO re pon e lines for stages A and C from CO_2 (rebreathing) tests At values below about 39 mm Hg (CO_2 threshold T) ventilation in stage A is apparently independent of the CO_2 ten ion and the ventilation CO_2 re pon e curve approaches a horizontal line (subthreshold ventilation)

Note the hift of the CO_2 re ponse line to the right with decrea e in wakefulne s (The broken horizontal line is the standard ventilation (sV) line)

were divided according to the slope, SQ, for stage A into 2 groups of equal number, the CO response curves of the group with the lower SQ had an sT_A of 38 9 ($\pm$ 0 6) and in the other group the value was 38 3 ($\pm$ 0 6) Therefore no apparent correlation was found between SQ and

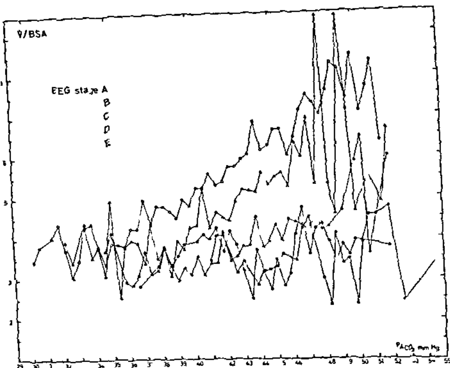


Fig 20 Mean ventilat on CO re ponse curves for the different stages of wakefulness of the CO_2 tests f om the first 3/4 of all the inve tigations (For details see text.) There is a clea shift downwards to the right of the response curves with decrease in wakefulness but there s overlapp ng between adjacent EEG stages

C *Mean ventilation CO_2 response curves during different levels of wakefulness*

In the \ P diagram in Fig 20 the \ P points" of the CO_2 tests (from the first three fourths of all the investigations) have been grouped in steps of 0 4 mm Hg of P_{ACO_2} and according to EEG stage of wakefulness Each point in the diagram hows the mean ventilation/m BSA for each EEG stage and each step of P_{ACO_2} Points belonging to corresponding EEG stages are joined to show the mean CO_2 respon e curves It is obvious from the diagram that the re ponse curves of adjacent stages of wakefulness over lapped However the diagram shows a clear tendency to a shift of the re pon e curve downwards to the right with a decrease in wakefulness

In Fig 21 the mean changes in position as well as in slope of the CO_2 re pon e curves with changes in wakefulness are given *quantitatively*

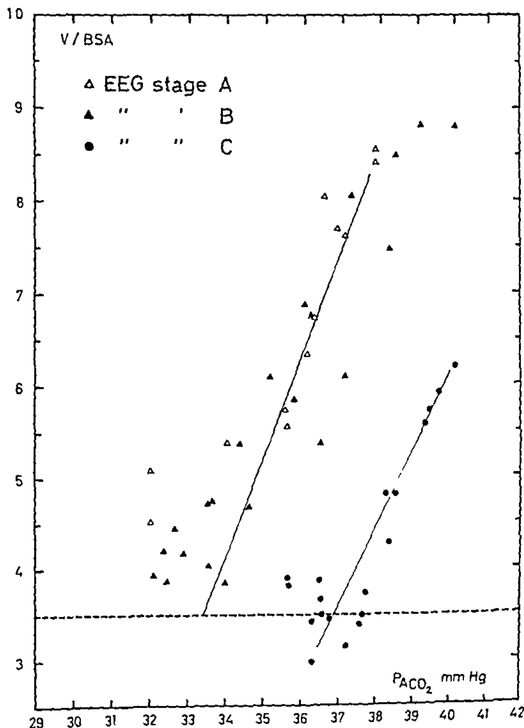


Fig 19 Subj B H male 23 years The diagram shows the same principal findings as in Fig 18 The shift between wakefulness and sleep of the CO_2 response line is here larger

This d a ram was constructed u the following way For all 73 separate investigations in which CO_2 tests were performed also during drowsiness and/or sleep the slopes (of the linear part) of the CO_2 respon e curves for wakefulness stage A were converted into one common reference mean slope This was obtained by multiplying $\dot{V}$/BSA of each V P po nt by th quotient [mean SQ/SQ for the individual] Then all stage A curves with a common slope were transformed into one common reference mean curve for all the investigations by adding to the P_{ACO_2} value of each V P point the difference in po ition (calculated in mm Hg at zero ventilation on the x axis of the V P diagram) as compared with the common mean curve

Then for each investigation the values for $\dot{V}$/BSA as well as for P_{ACO_2} of all the oth r V P points representing other stages of wakefulness were corrected by the same factors as those used for stage A The points in the diagram represent the means of the converted values for each step of 0 4 mm Hg for each EEG stage of wakefulne s (as in the diagram in Fig 20) The d agram is based upon more than 3 000 V P points Points during sleep situated clearly below the prevailin sT value were not included

In brief this means that all the response curves were scaled to one common reference curve (stage A) for all the individuals

It is evident from the diagram that there was a clear difference in po ition of the CO_2 response curve between wakefulness (stage A) and light to moderately deep sleep (stage C) or between stage C and deep sleep (stages D and E) For drowsiness (stage B) the curve was situated between stages A and C The scatter of the points was somewhat wider for drowsine s than for other stages a finding explained by the usually unstable respiration and EEG in this transitory stage between wakefulness and sleep

The diagram in Fig 21 also shows a slight decrease in the slope of the curves during sleep This is most obvious during the stages of deep sleep

The extension to the right of the response curves for sleep stages is limited in the diagram to about 50 mm Hg Therefore the curves for tages D and E are rather short In addition the number of V P points at the highest P_{ACO_2} values is relatively small during leep This is due to the fact that a P_{ACO_2} of 50 mm Hg or more was incompatible with leep, as mentioned in the foregoing chapter

The V P diagram in Fig 22 illustrates schematically the main differences de cribed between the CO_2 response curves of different EEG *stages*

The d agram also shows for each sta e the extrapolated "*apnoea threshold* (LINDHARD 1933) [I $_{CO_2}$ (ol ta ned by regression of the rectilinear parts of the curve *to the zero* l n of the y ax)

D *Ventilatory response to CO_2 during tense alertness*

During tense alertne s A_a there was a slight mean hift of the ventilation CO_2 respon e curve to the left (Figs 21 and 22) The mean D $T_{Aa\text{-}A}$ was

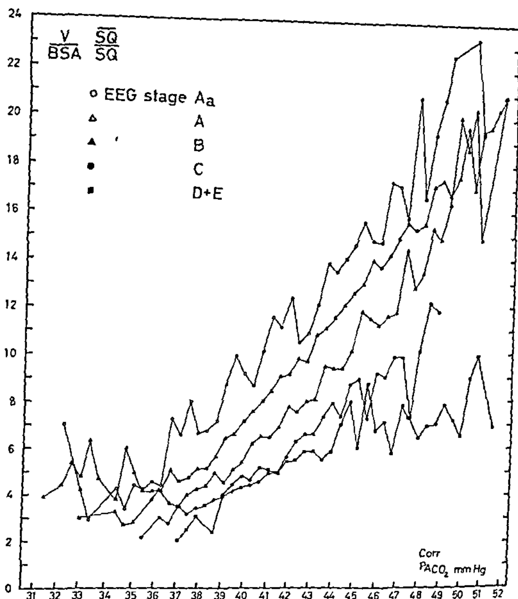


Fig 21 Mean ventilation CO_2 response of all CO_2 te ts from all the investigations (based upon more than 3 000 V P points) *The mean differences* in the po ition and slope of the re ponse curves between different levels of wakefulness (including stage A_a) are *here given quantitatively All points are scaled to one common reference curve stage A* For the calculation procedures and detailed comments see text

There is a clear shift downwards to the right but also a decrea e in the slope of the response curve with decreasing wakefulness For tense alertne a mean shift to the left is seen (without obvious change in slope)

This diagram was constructed in the following way For all 73 separate investigations in which CO_2 tests were performed also during drowsiness and/or sleep the slopes (of the *linear part) of the* CO_2 *re* pon e curves for wakefulness sta e A, were converted into one common reference mean slope This was obtained by multiplying V/BSA of each V P point by the quotient [mean SQ/SQ for the individual] Then all stage A curves with a common slope were transformed into one common reference mean curve for all the in vesti ations by adding to the P_{ACO_2} value of each V P point the d fference in position (calculated in mm Hg at zero ventilation on the x axis of the V P diagram) as compared with the common mean curve

Then for each investi ation the values for V/BSA as well as for P_{ACO_2} of all the other V P points repre enting other stages of wakefulnes were corrected by the same factors as tho e used for sta e A The points in the diagram represent the means of the converted values for each step of 0 4 mm Hg for each EEG stage of wakefulness (as in the diagram in Fig 20) The diagram is ba ed upon more than 3 000 V P points Points during sleep situated clea ly below the prevailing sT value were not included

In brief this means that all the response curves were scaled to one common reference curve (stage A) for all the individuals

It is evident from the diagram that there was a clear difference in position of the CO_2 re pon e curve between wakefulness (stage A) and light to moderately deep sleep (stage C) or between stage C and deep sleep (stages D and E) For drowsiness (stage B) the curve was situated between stages A and C The scatter of the points was somewhat wider for drowsiness than for other stages a finding explained by the usually unstable respiration and EEG in this transitory stage between wakefulne s and sleep

The diagram in Fig 21 also shows a slight decrease in the slope of the curves during sleep This is most obvious during the stages of deep sleep

The extension to the right of the response curves for sleep stages is limited in the diagram to about 50 mm Hg Therefore the curves for stages D and E are rather short In addition the number of V P points at the highest P_{ACO_2} values is relatively small during sleep *This is due to the* fact that a P_{ACO_2} of 50 mm Hg or more was incompatible with sleep, as mentioned in the foregoing chapter

The V P diagram in Fig 22 illustrates schematically the main differences de cribed between the CO_2 re pon e curves of different EEG stages

Th d gr m al o hows for each sta e the extrapolated apnoea threshold (LINDHARD 1933) of P_{CO_2} (obta ned by regres on of th rectiline r *parts of the curves to the zero* l ne of th x ax s)

D *Ventilatory response to CO_2 during tense alertness*

During ten e alertne s A_a there was a slight mean hift of the ventila tion CO_2 re pon e curve to the left (Figs 21 and 22) The mean $DsT_{Aa\ A}$ was

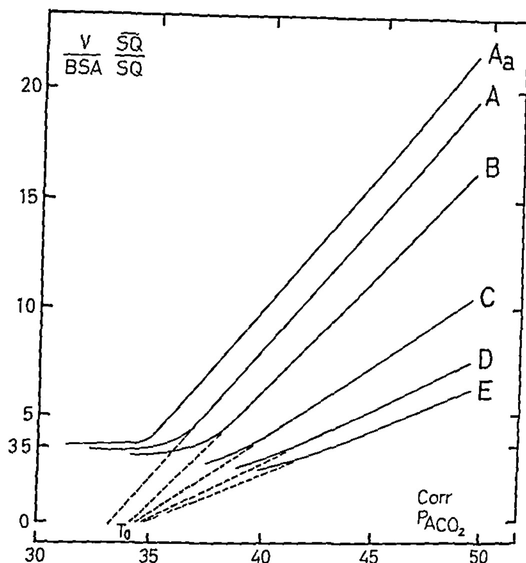
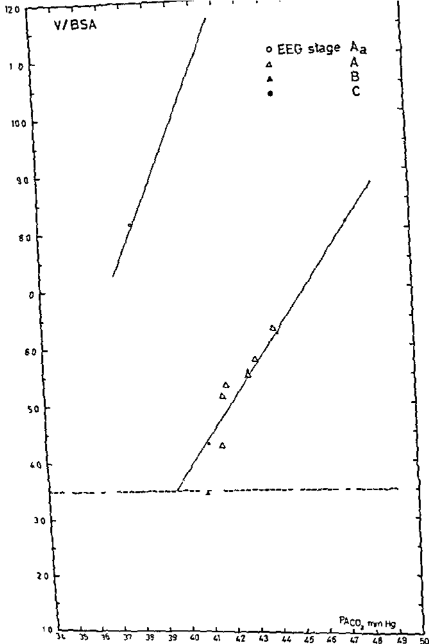


Fig 22 Schematic drawing showing the *principal findings from Fig 21* See text

1 4 ($\pm$ 0 2) mm Hg *It is evident from Fig 21 that the scatter of the values* around the ventilation CO_2 response curve was wide In 3 of the 30 investigations, where CO_2 tests were performed during stage A_a the position of the CO_2 response curve did not differ clearly from that for stage *A* In other investigations however the $DsT_{Aa\ A}$ was high and the SQ clearly increased (Fig 23) A typical very slight change is shown in Fig 24

There was no clear change observed in mean SQ when all the CO_2 tests of stage A_a were scaled to the common reference curve of stage A (Fig 21)

No correlation was found between the extent of the shift of an individuals response curve from wakefulness to sleep $DsT_{A\ C}$, and from wakefulness to tense alertness, $DsT_{Aa\ A}$



F g 3 Subj L H male aged ?? Ventilat on CO_2 re pon e lines for wakefulness (*stage A*) and f r *tense alertn ss* (*tage A_a*) There is a marked *shift to* the left and also increa ed l pe of the re pon l ne during sta e A_a in this case Us ally however the chan e was only l ght or moderate whithout any certain change in the slope

La ge symbols represent V P *points from* CO_2 *shunt tests*" (page 9) In this case the greater tability of the re p ratory balance during prolon ed CO_2 rebreathing had no clear infl ence on the posit on of the respon e line of stage A

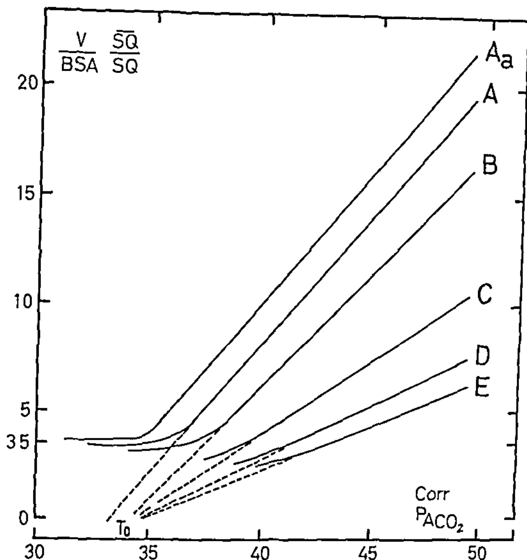


Fig 22 Schematic drawing showing the *principal findings from Fig 21* See text

1 4 (± 0 2) mm Hg It is evident from Fig 21 that the scatter of the values around the ventilation CO_2 response curve was wide In 3 of the 30 investigations, where CO_2 tests were performed during stage A_a the position of the CO response curve did not differ clearly from that for stage A In other investigations however the $DsT_{Aa\ A}$ was high and the SQ clearly increased (Fig 23) A typical very slight change is shown in Fig 24

There was no clear change observed in mean SQ when all the CO tests of stage A_a were scaled to the common reference curve of stage A (Fig 21)

No correlation was found between the extent of the shift of an individuals response curve from wakefulness to sleep $DsT_{A\ C}$, and from wakefulness to tense alertness $DsT_{Aa\ A}$

INTERINDIVIDUAL DIFFERENCES IN RESPIRATORY STABILITY DURING CHANGES IN WAKEFULNESS

A *DsT_{A-C} and periodic breathing*

In the preceding chapter it was shown that the change in respiration with level of wakefulness was particularly marked between stages A and C. This stage (C) of light sleep was noted in most of the subjects. The difference in 'standard threshold' of P_{ACO_2} between these stages, the DsT_{A-C}, was therefore used for comparison between individuals regarding respiration in wakefulness and sleep.

The variation in an individual's DsT_{A-C} on repeated occasions was usually small. The interindividual variation of the DsT_{A-C} was, however, considerable and ranged between 0.0 and 8.6 mm Hg.

Table VI (after text) gives the main data on SQ, sT_A and also the DsT between different EEG stages obtained during all investigations where CO_2 tests could be performed during stage A as well as during sleep and/or drowsiness. In addition, the degree of PB during onset of sleep in all these investigations is classified according to the following criteria:

+++ Very marked PB during usually repeated sections of at least 15 minutes duration. In the phases of hypoventilation $\dot{V}$ is for several breaths less than 1/4 of that during the hyperventilation phase. Sometimes even apnoea appears and the PB may closely resemble Cheyne Stokes breathing. The concomitant variations in alveolar CO_2 concentration is 1 per cent or more (this corresponds to P_{ACO_2} differences of more than mm Hg).

++ Prolonged PB (for more than 10–15 minutes) when $\dot{V}$ in the hypoventilation phases is less than about 1/3 of $\dot{V}$ during hyperventilation and the corresponding P_{ACO_2} variations are at least 5 mm Hg.

+ Distinct PB for at least 5–10 minutes but with less pronounced variation in $\dot{V}$ and P_{ACO_2}.

0 Very light or no PB.

A close agreement was found between the DsT_{A-C} and the degree of PB in all the subjects (Fig. 25). When the DsT_{A-C} of an individual was high, respiratory periodicity regularly occurred while he was falling asleep. Subjects with Cheyne Stokes' like breathing showed the largest DsT_{A-C}. Like the DsT_{A-C}, the type and degree of PB were fairly constant in each subject.

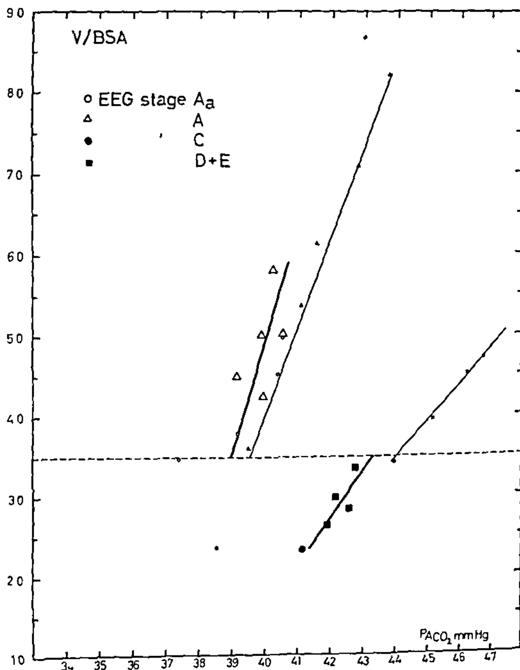


Fig 24 Subj I A female aged 22 This diagram illustrates a slight change in CO_2 response (shift of the curve to the left and probably also a slight increase in slope) during prolonged CO_2 rebreathing (CO_2 shunt tests see page 29) *Large symbols refer to* V P *values from* CO_2 *shunt tests* It is apparent that the *differences between wakefulness and sleep* in position and in slope of the response curve *are largely the same* during the routine CO_2 tests and during the prolonged CO_2 rebreathing at constantly increased P_{ACO_2} value

The former group was designated the '*unstable*' *group* because of the prominent lability in respiration with a change in wakefulness In contrast hereto the latter group was called the *stable* one

In some ca es it was not possible to calculate sT_C with accuracy This was usually due to too small a number of $\dot{V}$ P points during leep in 1 ca e because stage C passed so rapidly that CO_2 te ts for sleep were not practicable until during a deeper sleep stage In some cases only sT_B could be calculated It is clear from Table VI that like the DsT_{A-C} the $DsT_{A\ B}$ was higher in patients with marked PB Therefore such cases were grouped abo e or below 1 5 according to stability with the aid of SQ D $T_{A\ B}$

In a few subjects the EEG level of wakefulness invariably passed from stage A to stage C so quickly that sT could not be determined for a typical sta e B In these cases sT is given for the transitional stage B_1 between A and B (page 21)

When the subjects were divided according to $DsT_{A\ C}$ and not according to the product of SQ $DsT_{A\ C}$ only 1 borderline case (I S Table VI) was found to have changed from one group to the other It is thus clear that the variation in SQ (0 4 to 1 4) had no substantial influence on the degree of PB or a signment of a patient to the "unstable' or stable' group This was determined mainly by the $DsT_{A\ C}$ The $DsT_{A\ C}$ was in these normals thus not only representative of the change in position of the CO re ponse curve (increase of CO threshold value) but mainly also for the change in the ventilatory respon e to P_{ACO_2} between wakefulness and sleep

The two V P diagrams in Figs 26 and 27 are based upon the same values as in Fig 21 but separated according to the two groups of stability (Both groups thus are referred to the same reference curve of stage A) For the stable group there is only a small shift from wakefulne s to drow iness (stage B) in the po ition of the ventilation CO_2 re ponse curve The shift is however demonstrable with certainty for definite leep (stage C) Furthermore no definite decrea e in slope SQ is demonstrable in this group

The two groups of stability differed lightly in SQ and in sT_A *namely* for the former value 0 66 (± 0 05) and 0 85 (± 0 03) and for the latter value 40 1 (± 0 2) and 37 8 (± 0 4) re pectively (Table VI) The stable group showed the lower SQ and the higher sT_A The differences were however not ascertainable

C *Correlation between respiratory stability and other variables*

The two groups of respiratory stability were correlated with some other variable re piratory (measured during stable re piration) as well as non re piratory

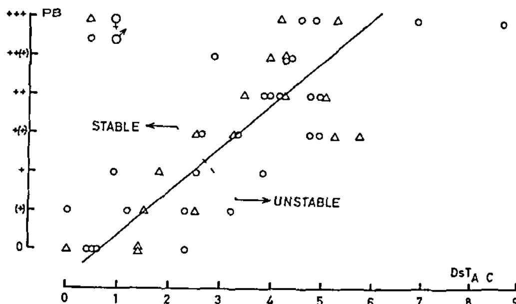


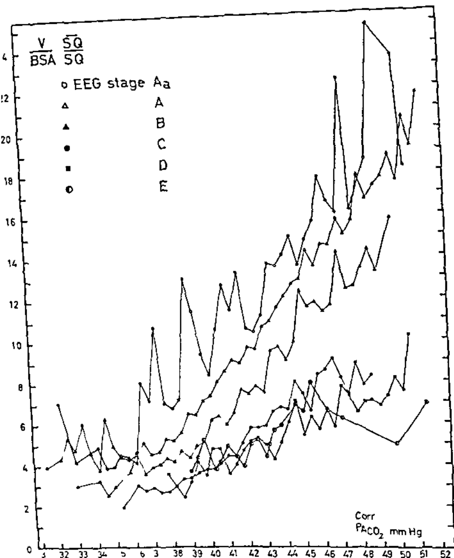
Fig 25 Correlation between degree of *periodic breathing (PB) and* magnitude of *shift of* CO_2 *response* curve ($DsT_{A\ C}$ in mm Hg) *between wakefulness and sleep* in all the 44 subjects in whom $DsT_{A\ C}$ was measured

In those subjects in whom $DsT_{A\ C}$ was measured also during reinvestigations the mean values are given In these cases the variations in $DsT_{A\ C}$ on different occasions were small (see Table VI) For grading criteria of PB see page 61

B *The "stable" and the "unstable" group*

In subjects with low SQ, a given shift in DsT implies a larger change *in ventilation than in subjects with a high SQ In order to assess the change in the ventilatory response to* CO_2, $DsT_{A\ C}$ was also multiplied by the subject's SQ This product, SQ $DsT_{A\ C}$ (also given in Table VI) might be supposed to reflect the degree of PB However the correlation to PB proved to be quite as good for $DsT_{A\ C}$ as for SQ $DsT_{A\ C}$

With the aid of SQ $DsT_{A\ C}$ the subjects were divided into 2 main groups (see Table VI), one characterized by disposition for PB when falling asleep, the other with at most a slight tendency to *PB* In the former group including slightly more than two thirds of the subjects the SQ $DsT_{A\ C}$ was larger than 2 5 At the onset of definite sleep, these individuals usually reached a larger difference in P_{ACO} as well as in V from the values when awake, also when breathing the ordinary air mixture (without carbon dioxide, Table VIII) In the deeper stages of sleep, too this group showed a larger increase in P_{ACO} than the other group The SQ $DsT_{A\ C}$ of the latter group varied between 0 0 and 2 5



F g Me n ntil t on CO_2 resp nse at d fferent levels of wakefulness of all unst ble" s bj ct (3 case 48 inve tig tions) As in F '6 the diagram is ba ed upon scaled al used for F g 1 Therefore the reference line (sta e A) has the same position and slope a n F gs 1 and 6

There i a p ominent shift downwa ds to the right and a clear decrease in the slope of th CO_2 re pon e curve with each decrea e in wakefulness The change between different level of leep i small r than between tages A B and C. For stage A_a there is large scatter of the po nt but only l ght overlapping of stage A

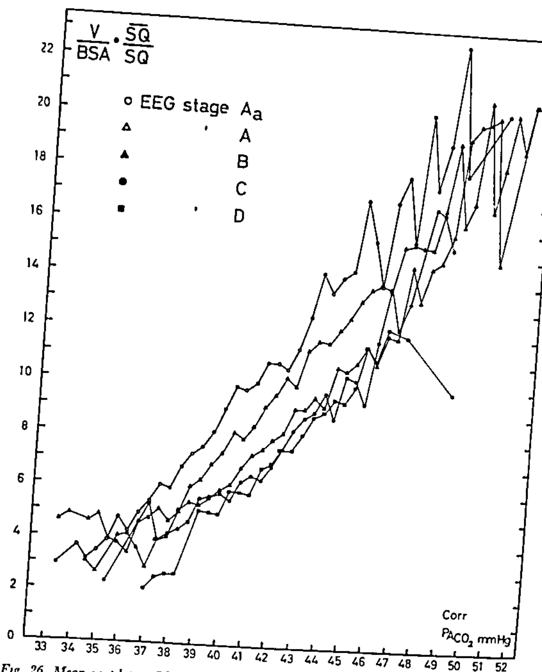


Fig 26 Mean ventilation CO_2 *response* at different levels of wakefulness *of all stable subjects* (18 cases 25 investigation) The diagram is founded on values calculated for Fig 21 with a common reference line for stage A

There is only a slight shift of the curve (downwards to the right) with decreasing wakefulness and no clear decrease in slope

Table VIII *Ventilation and* $P_{A_{CO_2}}$ *awake (stage A) and asleep (stage C) in the "stable" and the "unstable" group*

	V/BSA			$P_{A_{CO_2}}$			$\dot{V}_{stage C}$ / $\dot{V}_{stage A}$	$[\dot{V}/P_{A_{CO_2}}]$ stage C / $[\dot{V}/P_{A_{CO_2}}]$ stage A
	stage A	stage C	Diff	stage A	stage C	Diff		
Stable group (12 subjects)	3 4 (±0 2)	2 7 (±0 2)	0 7 (±0 1)	37 7 (±0 8)	39 8 (±0 7)	2 1 (±0 3)	0 80 (±0 07)	0 76 (±0 03)
Unstable group (17 subjects)	3 4 (±0 2)	2 5 (±0 1)	1 0 (±0 1)	36 8 (±0 4)	40 5 (±0 8)	3 8 (±0 4)	0 73 (±0 02)	0 66 (±0 03)

Both Table VII and Table VIII are founded on mean values from several separate V–P points in each subject and stage of wakefulness (stages A and C)

There was no noteworthy difference in any of the *pulmonary capacities* measured (vital capacity functional residual capacity and maximal breathing capacity) between the two groups of stability

The *body surface area* BSA was 1 8 (± 0 0) m^2 in both groups The two groups did not differ in respect of the *oxygen uptake* during a steady state in stage A (the basal metabolic rate) This was also the case regarding *age* and *sex* distribution (Table VI)

The two groups of stability were also compared as to differences in the EEG pattern during the routine examinations (page 10) Subjects belonging to the unstable *group* showed a stronger tendency to minor deviations from a very regular alpha pattern than did the stable cases (Bülow & Ingvar 1963 *c*)

Table VII *Tidal volume and respiratory frequency awake (EEG stage A) and asleep (EEG stage C) in the stable and the unstable group*

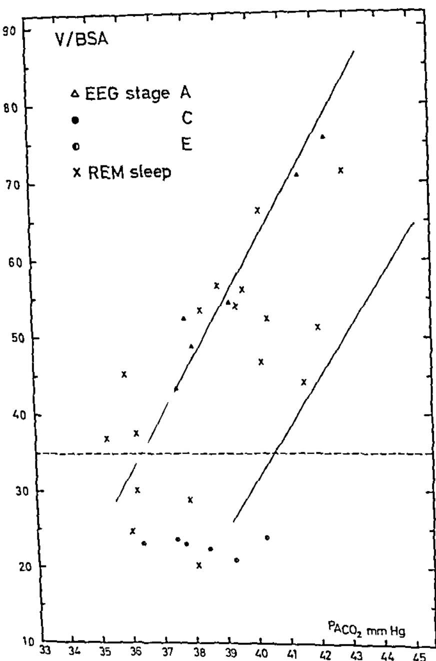
	V_T			f			$\frac{V_T}{f\ 10}$		
	stage A	stage C	Diff A−C	stage A	stage C	Diff A−C	stage A	stage C	Diff A−C
Stable group (12 subjects)	*490* (±40)	*310* (±20)	*150* (±30)	*13 0* (±0 6)	*15 0* (±0 5)	*−2 0* (±0 8)	*3 9* (±0 4)	*2 4* (±0 1)	*1 6* (±0 4)
Unstable group (19 subjects)	*430* (±20)	*310* (±20)	*110* (±20)	*15 4* (±0 9)	*15 1* (±0 6)	*0 3* (±0 6)	*3 0* (±0 3)	*2 2* (±0 1)	*0 9* (±0 [illegible])

Table VII gives the main data of frequency and volume of breathing in 31 *subjects in which a satisfactorily stable respiration occurred in sleep* during (oxygen enriched) air breathing In both groups the interindividual range of variation in *frequency of breathing* during wakefulness (stage A) was wide The mean ventilatory frequency was, however, lower in the stable group than in the unstable one 13 0 (± 0 6)/min and 15 4 (± 0 0)/min *respectively During sleep no difference in mean ventilatory frequency was* found between the groups (15 breaths/min in each group) Thus there was a mean increase in frequency of breathing between wakefulness and sleep in the stable group but an inconsiderable decrease in the unstable group

As to the *tidal volume* the decrease between wakefulness and sleep was clearly *present in both groups, 150 (± 30) ml/min) in* the stable group and 110 (± 20) in the unstable group

Thus in the subjects belonging to the stable group the decrease in tidal volume in sleep was largely compensated by the increa e in respiratory frequency resulting in only a small reduction of the total ventilation In the unstable group, however, the decrease in tidal volume during sleep was roughly proportional to the reduction in the ventilation Therefore the change in the quotient tidal volume/respiratory frequency was larger in the stable group

Table VIII summarizes the mean data for ventilation and P_{ACO} in stage A and C of the same subjects as in Table VII (except for 2 cases where not a sufficient number of accurate P_{ACO} values was available from a stable stage C) The *mean ventilation* during (oxygen enriched) air breathing and EEG stage *A was 3 4 (± 0 2) L/min/m² for* both the stable group and for the unstable one The corresponding values for *alveolar CO_2 tension* were 37 7 (± 0 8) and 36 8 (± 0 4) mm Hg respectively (The difference in P_{ACO_2} between the groups cannot be considered established)



F g 8 Subj I J female aged – *Ventilation CO_2 esponse est mation from REM sleep* For comparison the CO_2 response lines for stage A (awake) and C (light leep) are drawn There i a larg scatter of the V P point througout the CO_2 test durin REM sleep Howev r the me n CO_2 re ponse line see ns to be sit iated between the two lines in the diagram and therefore close to that for sta e B (For clarity the V P points from stage B also show ng a rather large scatter were excluded in the d agram.)

RESPIRATION DURING THE "RAPID EYE MOVEMENT" STAGE OF SLEEP

Electro oculography was performed in 14 of the investigations (on 12 subjects), simultaneously with EEG in order to detect the REM (rapid eye movement) stage of sleep Although periods of sleep of varying depth occurred during most of the investigations, only a few periods of REM sleep were long enough to permit detailed evaluation of the respiratory pattern and the ventilation CO_2 response

A *Respiratory pattern*

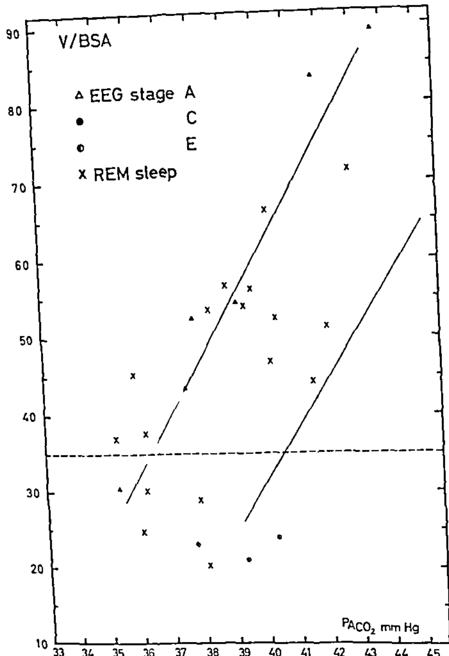
During all the periods of REM sleep a *pronounced respiratory instability* was observed In contrast to what was usually seen while the subjects were falling asleep, the respiratory variations during REM sleep appeared entirely irregular, without any obvious tendency to undulate as during PB

In REM sleep during air breathing V and P_{ACO_2} varied around the same mean values as in stage B There were no characteristic differences in the spirographic pattern of the individual breaths compared with stage B The striking general irregularity in respiration was particularly obvious when the eye movements were very frequent

Variations in the EEG pattern during REM sleep showed no close correlation with those in the pattern or degree of ventilation In general, however, when during this REM stage theta frequencies were prominent and the EEG pattern therefore resembled stage C ventilation was somewhat lower than during periods when low voltage fast activity dominated the EEG

B *Ventilation CO_2 response*

In some subjects episodes of REM sleep occurred during parts of CO_2 tests, but during other parts of the test the sleep stage was changed or in the absence of repeated eye movements not recognizable with certainty In at least 1 subject, however the main part of the CO_2 test was carried out during the REM stage of sleep (Fig 28) In this test there was a very wide scatter of the V P points The scatter was even wider than that for stage B of the subject The position and slope of the ventilation CO_2



F g 78 Subj I J female aged 2 I entil tion CO_2 *response estim tion from REM sleep* For c mp rison the CO_2 re pon e lines for stages A (awake) and C (li ht sleep) are drawn Th re i a large scatter of the V P points througout the CO_2 test during REM sleep Howev r the m an CO_2 r pon e line seems to be s tuated between the two lines in the diagram and therefore close to that for stage B (For clarity the V P points from sta e B also bowing a rather large scatter were excluded in the d agram.)

response curve were judged as largely the same as for stage B From the periods of REM sleep during CO_2 tests in the other subjects, nothing was found that argued against the assumption that *during REM sleep, the ventilation CO_2 response is of about the same magnitude as for stage B* However, the marked instability in respiration during REM sleep usually makes evaluation of the ventilatory response to CO_2 difficult

RESPIRATION AND WAKEFULNESS DURING BREATHING AIR WITH DIFFERENT CONCENTRATIONS OF OXYGEN

Control experiments were performed in order to study whether inspiration of the gas mixture with 40 per cent oxygen instead of the 21 per cent of room air implied any remarkable influence upon the main results presented In addition some measurements were made of the ventilation CO_2 response in wakefulness and in sleep during moderate hypoxaemia The measurements are few and therefore do not permit accurate quantitative evaluations

A *Inspiration of atmospheric air and of pure oxygen*

In 6 investigations (in 5 of the subjects) the oxygen concentration of the inspiratory air was altered for periods of 40 minutes up to a few hours from the usual level of 40 per cent either to pure oxygen (3 cases) or to the 21 per cent level of atmospheric air (3 cases)

In all cases the close interrelationship was preserved between the respiration and the EEG pattern The type and the degree of respiratory changes between the levels of wakefulness in an individual were the same at both oxygen concentrations So was the degree of PB The position and slope of the ventilation CO_2 response curve for a given individual and stage of wakefulness were furthermore largely the same whether he was breathing ordinary air pure oxygen or 40 per cent oxygen

B *Moderate hypoxaemia*

In 7 of the investigations the oxygen concentration in the closed system was successively decreased from that of atmospheric air to between 13—15 per cent (i e roughly 100 mm Hg P_{IO_2}) (This was obtained by shutting off the oxygen supply for about 10 minutes The oxygen concentration was then calculated from the volumetric changes in the system)

In 3 individuals the ventilation CO_2 response for both wakefulness and sleep could be measured from CO_2 tests when breathing 14 (13—15) per cent oxygen and compared with the results from breathing the ordinary 40 per cent oxygen during other parts of the same investigation period for each subject The results demonstrated that there was no substantial

response curve were judged as largely the same as for stage B From the periods of REM sleep during CO_2 tests in the other subjects, nothing was found that argued against the assumption that *during REM sleep, the ventilation CO_2 response is of about the same magnitude as for stage B* However, the marked instability in respiration during REM sleep usually makes evaluation of the ventilatory response to CO_2 difficult

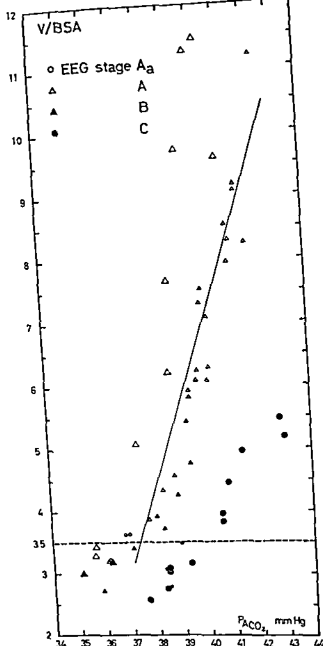


Fig 30 Subj I A f m le a ed 93 *Vent lation CO_2 r sponse d ring w kefulness and sleep when b eath g 15 per ent xygen* Large symbols refer to V̇ P points f om hypoxia. For leep (tage C) the same l kely tendency to a further decrease in CO_2 re ponse during h poxia as in Fig 9 s seen. During wakefulness (tage A) however there appears an incre se in the CO_2 r ponse during hypox a The change in position and slope is com parall to that for stag A_a in this subject.

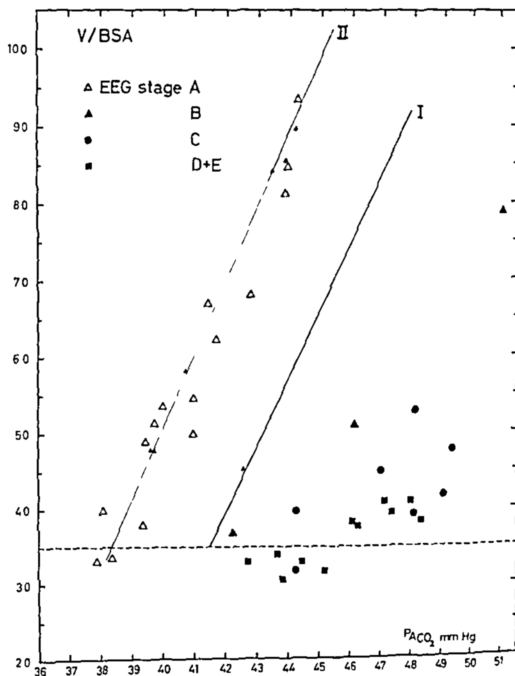


Fig 29 Subj S I male aged 21 *Ventilation CO_2 response during wakefulness and sleep when breathing 15 per cent oxygen* Large symbols refer to V P points from hypoxia All points along the stage A line marked I and to the right of this line refer to the first investigation on this subject No CO_2 test during hypoxia was then performed for stage A All points around stage A line II refer to a reinvestigation not preceded by deprivation of sleep In investigation I the shift in position as well as decrea e in slope of the CO_2 re pon e line is prominent during hypoxia (compare the small dots for the same sleep stage from breathing oxygen enriched air')

During wakefulne s (investig II) there is in this case no certain difference in CO_2 re pon e between hypoxia and O_2 air breathing

INFLUENCE OF 24 HOUR SLEEP—WAKEFULNESS RHYTHM AND OF SLEEP DEPRIVATION ON RESPIRATORY STABILITY DURING ONSET OF SLEEP

Some of the reinvestigations were performed in order to study whether the re piratory ' stability' during changes in wakefulness (Chapter VI) was influenced to any marked extent by the 24 hour sleep—wakefulness rhythm In some other reinvestigations the influence of sleep deprivation was likewi e studied

Of the regular night workers (all of which were examined in the morning after they had been on night work for several nights in succession) 3 subjects (I A B B and E N) were re examined during the night after they had slept during day time for several preceding days while 3 (S B A O and M F) were re examined in the forenoon after they had slept for several con ecutive nights

Among those subjects who were more occasionally on night work 3 (D F M A and B H) were re examined at the same time in the morning but after they had slept several nights in succession Of the non night worker 2 (A H and B I) were re examined in the night

It is clear from Table VI (page 107) that the intra individual variations of DsT and SQ in all 11 subjects were fairly small and of about the same order as in other reinvestigated subjects The series of the different exami nation conditions in question are small and allow no definite conclusions However *no obvious relationships were found between on one hand $DsT_{A C}$, SQ or sT and on the other hand the time of the investigation or the presence of sleep deprivation*

change in sT for identical sleep stages of an individual between breathing in 14 per cent and in 40 per cent oxygen This also holds for SQ for 1 of the subjects In the other 2 cases the SQ seemed, indeed, somewhat further decreased during the low oxygen breathing (Figs 29 and 30)

During wakefulness, stage A, SQ was moderately increased in the hypoxaemic state No prominent change in sT_A was observed In one of the *subjects the ventilation CO_2 response during hypoxia could also be com*pared to that during tense alertness (stage A_a) when breathing 40 per cent oxygen sT as well as SQ of this stage A_a was almost the same as that of stage A during the hypoxic state (Fig 30) *To conclude*, when the subjects were breathing about 14 per cent oxygen, the difference in SQ between wakefulness and sleep appeared slightly larger than when the arterial blood was saturated with oxygen In spite of a moderately increased ventilatory responsiveness to CO_2 during the hypoxaemic state when awake, no such change was recognized during sleep It should, however, be pointed out that the number of observations was small

INFLUENCE OF 24 HOUR SLEEP—WAKEFULNESS RHYTHM AND OF SLEEP DEPRIVATION ON RESPIRATORY STABILITY DURING ONSET OF SLEEP

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Of the regular night workers (all of which were examined in the morning after they had been on night work for several nights in succession) 3 subjects (I A B B and E N) were re examined during the night after they had slept during day time for several preceding days while 3 (S B A O and M F) were re examined in the forenoon after they had slept for several consecutive nights

Among those subjects who were more occasionally on night work, 3 (D F M A and B H) were re examined at the same time in the *morning* but after they had slept several nights in succession Of the non night worker 2 (A H and B I) were re examined in the night

It is clear from Table VI (page 107) that the intra individual variations of DsT and SQ in all 11 subjects were fairly small and of about the same order as in other reinvestigated subjects The series of the different examination conditions in question are small and allow no definite conclusions However *no obvious relationships were found between on one hand* DsT_A *c SQ or sT and on the other hand the time of the investigation or the presence of sleep deprivation*

CHAPTER X

RESPIRATION AND WAKEFULNESS IN SOME DISORDERS OF WAKEFULNESS (CONSCIOUSNESS) FUNCTION

The method described lends itself to clinical investigations It was used in some studies of respiration in disorders of the wakefulness (consciousness) function The investigations will be published separately (BULOW & INGVAR 1963 a and b, BULOW 1963 a, for references see these papers) Some other series are in progress, comprising the dumping syndrome and also heart and anxiety neurosis with respiratory symptoms Only a brief survey of the main findings in three of the series is given below, including patients with petit mal epilepsy, narcolepsy and the obesity cardiopulmonary syndrome ("pickwickian syndrome")

1 *Petit mal epilepsy* In the petit mal group (3 c/sec "spike and wave" epilepsy) it was found that the onset of the seizures does not primarily involve brain stem structures normally regulating wakefulness and respiration The seizures occurred whether the P_{ACO_2} was high or low Only an uncharacteristic usually very slight unevenness in ventilation was observed before, during, or after the attacks (BULOW & INGVAR 1963 a) The normal linkage between respiration and wakefulness was preserved

The ventilatory response to CO_2 varied within normal limits as did the change in the response during sleep A close analysis of seizures occurring during CO_2 rebreathing tests did, however reveal a definite increase in the ventilation CO_2 response *during* the seizure episodes The results implicate that an increased "CO_2 sensitivity" develops during petit mal attacks (BULOW 1963 a) During (oxygen enriched) air breathing at rest the change in sensitivity could not be demonstrated since the CO_2 drive on ventilation was then usually absent At low CO_2 tensions there was a reduced ventilation at very low tensions complete apnoea appeared shortly after the onset of the seizure

2 *Narcolepsy* It was demonstrated that the imperative attacks of drowsiness and sleep—with the corresponding normal sleep changes in the EEG pattern—characterizing the narcolepsy syndrome, are regularly accompanied by changes in respiration in the typical manner described here for normals Thus the narcoleptic episodes did not differ qualitatively

from what normally occurs during periods of onset of sleep (BULOW & INGVAR 1963 b) The sleep attacks were only more rapid and frequent than what was observed in normals under similar experimental conditions P_{ACO} was normal when the patients were awake as judged from the EEG pattern No abnormalities in CO_2 regulation of respiration were observed (BULOW 1963 a)

The results supported the opinion that—contrarily to what was observed in petit mal epilepsy—a primary defect is situated within regions of the reticular system participating in both wakefulne s and respiration regulation

3 *Obesity cardiopulmonary syndrome* As in the two above mentioned group the normal linkage between respiration and wakefulness function was preserved in the three cases studied which clinically belonged to the obe ity cardiopulmonary or pickwickian ' syndrome In this group which is also characterized by hypoventilation a periodic somnolence was found which was concomitant to a very marked periodic breathing closely re embling CHEYNE STOKES breathing In one of the patients with narco lepsy who e symptoms were very marked similar results were obtained Some further findings in common for the two groups narcolepsy and the pickwickian syndrome suggested a weakness in both groups of a mecha ni m in the brain stem as the cause of the disturbance of wakefulne s This in turn contributes to the development of periodic breathing (BULOW 1963 a)

Pat ents with obe tas but without the cardiopulmonary *syndrome* were al o studied *In ca es* whithout any obvious disturbance of wakefulness regulat on the respiratory variat ons during changes in wakefulness showed no tend ncy to deviation from the normal ran e In two other obese subjects who had minor signs and symptoms of wakeful ness distu bance a prominent re p ratory periodicity was pr sent during *variations in* wakefulness These observations are howe er at present too limited to admit definite conclu ions

In conclusion the present technique which allows evaluation of the ventilation CO_2 response even for rather brief periods as in petit *mal* or narcolepsy attacks (which u ually last for only a minute or le s) has enabled elucidation of pathological events presumably occurring within the reticular system of the brain stem

DISCUSSION

In the present investigation changes between different levels of wakefulness in the EEG were correlated with respiratory variables Two approaches were used Firstly, spontaneous changes in the variables were studied Secondly, a load was put on the respiration (during the CO tests) at various levels of wakefulness, in order to examine the responsiveness of the respiratory centres during wakefulness and sleep

In the following discussion attention will be given first to methodological questions including the choice of methods for measuring respiration and the ventilation CO_2 response at various levels of wakefulness

THE METHODS USED

Carbon dioxide sensitivity In the present investigation the term "CO sensitivity" refers to the responsiveness of the respiratory centres in a broad sense of the term *i e the respiratory control system* including all cerebral structures specifically sensitive to CO_2 as well as all peripheral receptors participating in the respiratory regulation It is not possible to say to what extent these different parts influence ventilation and the arterial carbon dioxide tension under the conditions used in the present study It is, however, commonly believed that the *peripheral chemoreceptors*, sensitive to CO_2, in the circulatory system, normally play a subordinate role in the respiratory regulation (GESELL *et al* 1940 SCHMIDT & COMROE 1940, HESSER 1949, KAO *et al* 1962) The activity of these receptors cannot be measured directly in man According to G C LOESCHCKE (1953) such receptors are responsible for about 14 per cent of the total CO_2 drive of ventilation in man (*cf* HEYMANS & NEIL 1958)

CO_2 and H^+ The aim of the present work was not to evaluate the part played by the hydrogen ion H^+ in the ventilatory regulation Since the changes in arterial P_{CO_2} between wakefulness and sleep were obviously caused by the changes in total (and alveolar) ventilation the change in arterial H^+ (or pH) must be considered secondary to the changes in P_{ACO_2} Also during the CO_2 tests the increase in H^+ drive on ventilation is dependent upon the increasing P_{ACO_2} Under these conditions the variations in P_{CO_2} and H^+ may be roughly parallel As their effects on ventilation are commonly considered to be additive to each other (GRAY 1950 NIELSEN & SMITH 1952) distinction between the two factors seemed to

be of le s importance in the pre ent investigation The stimulus studied may be properly defined as *les propriétés du sang liées à CO_2* (DEJOURS et al 1958)

For practical purpo es the *alveolar* P_{CO} was measured instead of the arterial P_{CO_2} Capnography permits measurement of even rapid breath to breath variations In Chapter II control experiments were described which showed a parallelism between the expiratory end plateau P_{CO_2} and the arterial P_{CO} the absolute capnographic value being about 3 mm Hg below that of the arterial blood The end expiratory P_{ACO} can therefore be used as an *index* of the CO_2 tension acting upon the respiratory centres The dependence of the true ' CO_2 ten ion on the cerebral blood flow will be discus ed later

It may be recollected that even small and tran ient ventilatory changes are recognizable in the arterial blood with the aid of continuous measurements of arterial pH and P_{CO_2} (HESSER 1949 GLEICHMANN & LÜBBERS 1960 BJURSTEDT et al 1962)

The *response to the CO_2 stimulus* was measured from the total ventilat on There are surely se eral intermediate links in the chain from the re piratory centres in th brain stem via the efferent neuronal pathways the neuromuscular junction, the re piratory muscles and finally to the ventilatory air flow which was measured during each breath. Recording of the electric acti ity in the phrenic nerve has been exten ively used in animal exper ments Electro-myographic re ording from the respiratory muscles represents measurement at a site nearer to the target of the stimulus However electromyography measures the activity of the ventilatory muscles in relative terms only Furthermore the method is painf il and it could therefore not be used in the present investigation There ar also methods for mea uring of the ventilatory work as well as its oxygen co t (CAMPBELL et al 19 7 BRODOV KY et al 1960) They are however not accurate enough It was the efore decided to use measurement of the ventilation in the present st dy

The question whether *total* or *alveolar ventilation* should be used in a study of the pre ent type should also be considered Total ventilation seems to provide a better mirror of the end result produced by the neuronal volleys to the re piratory mu cles (LINDHARD 1933 NIELSEN 1936 SCHAEFER 1958 LAMBERTSEN 1960 LOESCHCKE 1960) The alveolar ventilation and the alveolar CO_2 content together provide a direct measure of the pulmonary CO_2 output and during a steady state also of the total body metabolism (*cf* GRAY *et al* 1956 BELLVILLE & SEED 1960) During such a steady state the correlation between the stimulus index P_{ACO_2} and V_A is pre umably clo e The frequent changes in rate and volume of breathing observed may however influence the ratio between alveolar and total ventilation Furthermore at present *no* method is available by which breath by breath variations in the alveolar ventilation can be measured as accurately as the total ventilation can be in spirography

It should be pointed out that only *concomitant* measurement of both respiratory frequency and volume can give accurate information on the degree of ventilation As mentioned above both values normally vary

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ness There is general agreement that these stages are easily identified from the EEG tracing by the trained eye (*cf* GIBBS & GIBBS 1951) The main stages of wakefulness drowsiness and sleep used here show good agreement with behavioural tests and also with the increase in the arousal threshold to auditory stimuli (JUNG 1954 DEMENT & KLEITMAN 1957 ROTH 1962 FISHGOLD & SCHWARZ 1961 OSWALD 1962 and others)

It should be observed however that it is sometimes difficult to classify EEG patterns recorded during transitions from one level of wakefulness to another This may partly be attributed to individual but also to age differences in the EEG pattern. Such transitional stages have for reasons outlined elsewhere (page 78) received much less attention in the quantitative analysis

Frequency analysis enables quantitative resolution of the EEG pattern This method is, however of a very limited value during rapid variations in the EEG pattern as e g during onset of sleep (FISHGOLD & SCHWARTZ 1961)

The "rapid eye movement (REM) stage of sleep is identified by simultaneous use of electrooculography and EEG (DEMENT & KLEITMAN 1957 SCHWARTZ 1962)

Other methods for estimation of level of wakefulness comprise recording of various autonomic functions such as electrodermatography (galvanic skin response) plethysmography (*cf* NILSSON 1960) arterial blood pressure heart frequency as well as electromyography and measurements of body movements and body temperature

Among these methods the electric resistance of the skin has proved to be correlated to some extent with EEG stage of sleep (JUNG 1954) including REM sleep (HAWKINS *et al* 196). Electromyographic findings on the other hand are difficult to correlate with sleep stage since the activity of different muscles is often largely dependent upon changes in posture During REM sleep there does not seem to be such a good correlation between the EEG and the activity of neck muscles in man as in animals (JOUVET *et al* 1960)

A state of *tense alertness* (stage A_a) is for obvious reasons difficult to control and to uphold at a constant level It is also difficult to judge from the EEG even in experiments including analyses of kappa rhythm' (CHAPMAN *et al* 1962) The respiratory measurements from stage A_a therefore only permit crude estimations

TECHNICAL CONSIDERATIONS

EXPIRATORY END PLATEAU P_{ACO} MEASUREMENT DURING SLEEP

No increase in *dead space* was observed during sleep but rather if any thing a very slight decrease The ratio V_D/V_T was largely unchanged This suggests that sleep is not accompanied by any noteworthy change in the ventilation/perfusion ratio for different parts of the lung Furthermore the spirographical end tidal level was largely unchanged indicating a stable functional residual capacity Consequently also during sleep the expiratory end plateau measurement of P_{ACO_2} was considered reliable as were changes

widely Furthermore, in the series investigated, changes in frequency and in tidal volume proved to be prominent during *shifts in wakefulness It is* thus not possible to use pneumographical measurements of chest wall *movements or thermo sensitive* measurements of ventilatory air flow (*cf* MARTIN 1960) These methods only give a qualitative measurement of the ventilation

Reliable measurement of the ventilatory *response to CO_2 requires many* V—P points (page 26) corresponding to ever increasing P_{ACO_2} (JULICH 1951, PAULI et al 1959) Careful determination of the response curve by a substantial number of points turned out to be definitely necessary for recognition of slight shifts in the position or in the slope of the curve A stepwise increase of P_{ACO_2} by *administration of gas mixture with* increasing amounts of CO_2 would have entailed undue extension of the time necessary for a complete test Every step would have required a long time for the development of a steady state

The main reasons for using *CO_2 rebreathing* in the CO_2 tests were given in the final *paragraph under 'Methods'* It was stressed that during undisturbed whole night sleep regular, rhythmical and prominent undulations occur between the EEG stages of sleep (ASERINSKY & KLEITMAN 1953 DEMENT & KLEITMAN 1957, SHAPIRO 1962) These variations were always found to be accompanied by respiratory changes too Obviously therefore, it is *not possible to place stringent demands on a complete* respiratory 'steady state' for the repeated estimation of the ventilation CO_2 response curves at various levels of wakefulness

It was shown (Method page 29) that the increase rate of P_{ACO_2} with the CO_2 test used provided a satisfactory dynamic balance in the ventilation P_{CO_2} *relationship, permitting comparison* between individuals Such comparison was, however, not possible for transient periods of marked changes in wakefulness with concomitant marked respiratory instability

The rate of increase in P_{ACO_2} during CO_2 tests was not substantially changed between wakefulness and sleep (The decrease in ventilation during sleep tended to increase the rate whereas the decrease in $\dot{V}_{ECO_2}$ had the opposite effect by reducing the increase in inspiratory P_{CO_2})

The CO_2 tests permitted rapid and repeated measurements of the ventilation CO_2 response for various levels of wakefulness during each investigation period The number of available V—P points thus became sufficiently large to yield response curves (and their sT values) at various EEG levels Furthermore in order to avoid CO_2 induced arousal reaction the CO_2 rebreathing could easily be interrupted whenever the EEG record showed signs of disturbed sleep

Evaluation of the EEG Neither the level of wakefulness nor the 'depth of sleep' can be quantitatively defined The EEG is however at present the most reliable method for classifying separate stages of wakeful

output The following principal observation during these respiratory changes has to be stressed

When following a decrease in wakefulness successive V—P points" during a CO_2 test were plotted in a V—P diagram they fell around the ventilation—CO_2 response curve of the prevailing EEG level *The scatter along the response curve was not found to be substantially dependant on deviations from a respiratory steady state* The gradual readjustment of the two respiratory variables V and P_{ACO_2} entailed a shift upwards to the right but largely along the same CO_2 response curve

Within half an hour or so a slight shift sometimes occurred in the position of the CO_2 response curve Evaluation of the course of this late and always moderate adjustment is beyond the scope of the present study There are various regulation mechanisms—humoral, circulatory and neuronal—secondarily involved in the final adjustment of adaptation of respiration at a new level of equilibrium such as the one during uninterrupted sleep This combined effect was studied by the prolonged CO_2 shunt tests" It should be recalled that the change in position of the CO_2 response curve between wakefulness and sleep the $DsT_{4.C}$ was then largely unchanged (page 29)

Deep sleep generally takes a long time to develop It is a matter of conjecture whether the comparatively small respiratory variations during changes between deeper sleep stages may not be partly attributed to the fact that adaptive mechanisms of the type discussed above have had more time to develop However even the EEG changes between deep sleep stages (D and E) are usually less striking than between stages A B and C. The true" change in wakefulness between the EEG stages of deep sleep (D and E) is perhaps also relatively small

To conclude measurements of the ventilation CO_2 response at different levels of wakefulness were largely unaffected by deviations from a respiratory steady state in the strict sense of the word

It should be pointed out that for marked *arousal reactions* or periods immediately following them no attempt was made to evaluate the ventilation CO_2 response The concept of level of wakefulness" is difficult to apply during such events characterized by rapid marked changes in various functions as well as in the EEG pattern (JUNG 1954)

TIME DELAY BETWEEN A CHANGE IN P_{ACO} AND IN VENTILATION

The 25 second delay measured in the present study (page 25) represents the lag noted for the initial CO_2 effect upon ventilation caused by a moderate and transient change *in alveolar* P_{CO_2}

This delay is not identical with the time necessary *for a* stabilized (optimal) ventilatory change to a permanent change of the CO_2 *stimulus* According to HESSER (1949) and ÅSTRÖM (19) the complete ventilatory response of the medullary respiratory centres requires some minutes which is of the same *order as in* measurements of the ventilatory response to gas mixtures containing fixed CO_2 concentrations (cf NIELSEN 1936)

in P_{ACO_2} between wakefulness and sleep This conclusion was supported by the control measurements of arterial and mixed venous P_{CO_2} (page 22)

During the CO_2 tests rebreathing of higher CO_2 concentration than about 2 5 per cent was accompanied by a reduction of the difference between P_{aCO_2} and P_{ACO_2} Presuming a normally occurring admixture of some (alveolar?) dead space to the end plateau portion as being the cause of the a—A difference in P_{CO_2} observed this difference should decrease when CO_2 is added to the inspiratory and consequently also to the dead space gas This might cause a slight decrease in slope SQ of the CO_2 response curve Assuming an SQ of 1 1 and an a—A difference of 2 5 mm Hg at the beginning and of 1 75 at the end of the CO_2 test (when the inspired CO_2 content had increased from 0 to 5 per cent) an observed increase in P_{ACO_2} of 6 5 mm Hg during the test would then imply an increase in P_{aCO_2} of 5 75 mm Hg If the slope SQ is calculated from this true change in arterial P_{CO_2} it should be corrected from 1 1 to 1 2(4) It should be stressed however that such an error would not noticeably influence the relative changes in slope (SQ) from wakefulness to sleep since the decrease in a—A difference during a test was about the same on repeated tests in a given individual irrespectively of level of wakefulness The sT values would not be affected since they were measured from the very first part of the response curves (when the inspiratory CO_2 content was below 1 per cent)

The decrease in the a—A difference for P_{CO_2} during a CO_2 test might be expected to be somewhat different between individuals according to their a—A difference when breathing the CO_2 free gas mixture However as found in the controls with arterial P_{CO_2} (page 23) the decrease in a—A difference was far less than what would have been expected if it was caused only by dead space admixture This observation is further supported by a recent report by HAAB & PIIPER (1961) who found that only half of the gradient between end expiratory and arterial P_{CO_2} in dogs could be ascribed to the alveolar dead space admixture the other half remaining unexplicable

In conclusion the reduction in the a—A difference during CO_2 tests apparently had only a very little influence on the SQ measured

The action of a moderately increased amount of CO_2 on the pulmonary airways and gas distribution as well as blood flow, in normal man has not been satisfactorily clarified According to FISHMAN et al (1960), such changes during inhalation of 5 per cent CO_2 are slight and without any definite influence on pulmonary vascular resistance and blood flow Consequently the local pulmonary action of CO_2 may be neglected in the present measurements of the ventilation CO_2 response

RESPIRATORY EQUILIBRIUM

In the present study there were sudden transient, as well as more or less gradual respiratory changes which occurred throughout every recording period They were either concomitant with changes in wakefulness or caused by CO_2 rebreathing Following a fall in wakefulness, a discrepancy often appeared between 1) the largely unchanged metabolic production (as measured from the O_2 uptake) and 2) the decreased ventilatory output of CO_2 This was followed by a gradual restitution of an appropriate CO_2

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According to ÅSTROM (1952), the peripheral chemoreceptors react promptly — within a few seconds — to a change in pulmonary arterial oxygen tension Considering this, it seems likely, in contrast to a recent report by BOUVEROT *et al* (1962), that the CO_2 action is mediated mainly via the central nervous chemoreceptors (*cf* DEJOURS *et al* 1958) It should, however, be recollected that a delay caused by the circulation time lung cerebrum (ear) is only about 6—7 seconds (BROBEIL *et al* 1957)

In a few cases 7 per cent CO_2 was given during 2 or 3 breaths to sleeping subjects No obvious change in the time delay could be recognized compared whith that in wakefulne

MECHANICS OF THE LUNGS AND THORAX DURING SLEEP

When judging the reliability of the measurement of the ventilatory response, the mechanics of the lungs and the thorax must also be considered The present spirographical technique had only a negligibly small influence upon the respiratory variables measured The added instrumental dead space was small and so was the resistance to the rather low maximal ventilatory flow rate during the present experiments

ROBIN *et al* (1958), in 3 normal subjects found no clear change in pulmonary compliance but a slight decrease in air way resistance during sleep In view hereof, the observed decrease in slope of the CO_2 response curve during sleep might possibly have been slightly underestimated in the present study However, a decrease in air way resistance must be much larger if it is to cause a noticeable increase in slope of the response curve

HYPOXIC DRIVE ON VENTILATION

By maintaining inspiratory oxygen concentration at about 40 per cent any noteworthy hypoxic drive of ventilation was avoided (LOESCHCKE & GERTZ 1958) In addition, at this moderate oxygen concentration, the increased ventilation during high oxygen tension (YAMADA 1918, SHOCK & SOLEY 1940 LAMBERTSEN *et al* 1953), seems not to have been of any importance

CEREBRAL BLOOD FLOW

In the discussion above it was stressed that the alveolo arterial P_{CO_2} was used only as an index of the stimulus of the ventilation—CO_2 response measured It certainly cannot be concluded that this CO_2 tension is identical with that acting upon the respiratory centres

LAMBERTSEN *et al* (1953) and LANDMESSER *et al* (1957) have claimed that preference should be given to measurements of the cerebral venous instead of the arterial P_{CO_2} in studies of ventilation P_{CO_2} response For the

venou P_{CO} hould more closely reflect the cerebral ti ue P_{CO} It seem however that this view needs reconsideration Recent work on carbon dioxide diffusion (GLEICHMANN *et al* 1962 SIESJO 1962) has clarified the relation hip between the arterial mean tissue and venous P_{CO} of the cerebral cortex The differences between these values are dependent on the regional blood flow and regional metabolic rate the tissue P_{CO} being approximately the mean of the corresponding arterial and venous P_{CO} Little is known of the regional blood flow in the brain stem (*cf* SOKOLOFF 1961) It seems at present not po sible to judge whether the ti ue P_{CO} of the re piratory centres lies closer to the arterial or to the cerebral mean venous P_{CO_2} of the internal jugular vein

In leep the mean cerebral metabolic rate is mainly unchanged (MANGOLD *et al* 1955) It might be suppo ed that this also holds true for the re piratory regulating structures It is not known whether the probably direct vaso dilatory action of CO_2 on the cerebral vessels (SCHMIDT 1950 SOKOLOFF 1959) is influenced by sleep wakefulness changes Provided there is no uch influence of any importance the increase in the P_{ACO} during sleep may most likely be the cau e of the roughly 9 per cent increase in cerebral blood flow ob erved by MANGOLD *et al* (1955) This increased blood flow would then slightly reduce the cerebral arterio venous and artery to ti sue P_{CO} differences When therefore during sleep the arterial and the cerebral tissue P_{CO} approach each other with increasing tensions this al o means that the ventilation — cerebral tissue P_{CO_2}" re ponse curve is not only situated omewhat to the right of the alveolar P_{CO} response curve measured but that it is also somewhat steeper During sleep this difference in po ition should be reduced by about 0 7 mm Hg assuming a cerebral arteriovenous P_{CO_2} difference of 15 mm Hg and a 9 per cent increa e in blood flow

It should be tre ed that the considerations et forth above do not take into account any pos ible change during leep in the proportions between the CO_2 effect on the central and on the peripheral chemoreceptors

CO_2 *threshold*

In several individual the CO thre hold (T) for a ventilatory response to P_{ACO_2} (NIELSEN & SMITH 1952 LOESCHCKE & GERTZ 1958) was not exceeded during air breathing During CO_2 rebreathing this threshold was present whether arterial or end expiratory P_{CO_2} was measured and even during a re piratory steady state (during CO_2 hunt tests") The threshold phenomenon cannot therefore be explained solely by an increased ad mixture of dead space to the end expiratory portion at low tidal volumes

The reaction time of a ventilatory response to a change in P_{ACO_2} was of a fairly constant magnitude It was not related to the threshold phenomenon since the difference between the P_{ACO_2} value at the onset of a CO test and that expressing the CO_2 threshold T varied widely The difference was sometimes very slight or non existent CO_2 rebreathing then gave an approximately rectilinear ascent of the response curve within half a minute from the onset of the test This finding seems not to be compatible with the recent suggestion of RILEY *et al* (1963) and of ARMSTRONG *et al* (1961) who proposed a CO_2 drive on ventilation mediated mainly by chemoreceptors in the pulmonary arteries Such a mechanism would have caused a longer time delay from the onset of CO rebreathing until a ventilatory increase took place

There are thus reasons to believe that in many subjects (breathing air) the carbon dioxide tension of the blood plays only a subordinate role in the ventilatory regulation (BOOTHBY 1912, MILLS 1946, RAMSAY 1959, FINK 1961) Since hypoxic drive on ventilation was avoided in the present study the blood gases may be excluded as the cause of this "subthreshold" drive on ventilation The problem will be considered further below

In view of the above discussion (pages 81—86) it may here be concluded that the observed decrease of the ventilatory response to CO_2 (including the change in threshold and slope) during sleep cannot be ascribed to methodological or technical errors

GENERAL COMMENTS

1 VENTILATION AND WAKEFULNESS

The results clearly demonstrate that in normals any decrease in wakefulness (with disintegration of alpha rhythm and appearance of theta waves in the EEG) is always accompanied by a decrease in ventilation During arousal the opposite change takes place Some preliminary observations in a small group of normal subjects (BULOW & INGVAR 1961) have thus been confirmed The present series shows that these findings have general validity, even for very transient changes in wakefulness, during a few seconds or less In general the changes in ventilation showed a very close time relation to the EEG changes the P_{ACO} changes always following more gradually as an expression of the simultaneous change in the alveolar ventilation and the CO_2 output The EEG changes during arousal from deep sleep, however, were observed to precede the ventilatory reaction by one second or two

The findings include definite differences in respiration even during stabilized respiratory conditions ("steady state") between various levels of decreased wakefulness Furthermore an increase in ventilation is observed with an increase in level of wakefulness during tense alertness (see further page 95)

The findings summarized above give further support to the assumption that the brain structures which regulate wakefulness (MORUZZI & MAGOUN 1949 MAGOUN 1952 DELL 1952 HUGELIN 1956) and the "cortical excitatory state" (JASPER 1936 *cf* BONVALLET *et al* 1954) (as reflected in the EEG) and those commonly called the respiratory centres (PITTS 1946 WYSS 1954 LILJESTRAND 1958 SALMOIRAGHI & BURNS 1960 and others) are closely functionally interrelated The existence of such a relationship is supported by much recent work on the functional organization of the so called reticular system (BONVALLET *et al* 1955 ROSSI and ZANCHETTI 1957 and others *cf* also HESS 1938 and INGVAR 1958)

2 VENTILATION CO_2 RESPONSE ("CO_2 SENSITIVITY") DURING SLEEP

A shift of the response curve during the CO_2 tests does not necessarily imply a change in the sensitivity of the respiratory control system to the very testing agent the CO_2 The CO_2 sensitivity of the specific chemoreceptors might be unchanged The decreased ventilatory responsiveness as manifested by the increase of the CO_2 threshold during sleep may be caused by a reduction of various other ventilatory stimuli neuronal *or humoral* exerting a direct or indirect action upon the respiratory centres within the brain stem The role of the proprioceptive afferents (from the body as a whole and from the respiratory muscles) should here be kept in mind (see further below page 88)

It seems more likely that the decrease in the *slope* of the ventilation CO_2 response curve during sleep should represent a real change in CO_2 sensitivity (GRAY 1950) Even this change in slope may however be caused by a reduction of other stimuli which interfere with or modulate the action of CO_2 upon the respiratory centre

It should be pointed out that not only the change in ventilation but even the *change* in ventilatory response to CO_2 developed very rapidly during a change in wakefulness (*cf* page 53) Such a rapid change is most likely of neuronal nature It cannot be explained by an acute change in total body or total cerebral metabolic rate

The change in cerebral blood flow during sleep (MANGOLD *et al* 1955) is as mentioned above probably secondary to the increase in arterial P_{CO_2} Changes in humoral factors in the blood stream affecting respiration such as catecholamine hormones and electrolytes may all be presumed to have a comparatively slower action on the respiratory control system

Thus the present indirect study of the ventilation CO_2 response ("CO_2 sensitivity") does not permit any definite conclusions concerning the site and the mode of the change in respiratory action of CO_2 during sleep ...

present evidence favours mostly a neuronal mechanism Brain stem structures, sensitive to CO_2 (VON EULER & SODERBERG 1952 a and b) may be suggested as the primary site of this mechanism

THRESHOLD AND SLOPE OF THE CO_2 RESPONSE CURVE DURING SLEEP

Previous investigators have found a prominent decrease in "CO sensitivity" during sleep (MAGNUSSEN, 1944, in 2 cases, ROBIN *et al* 1958, 3 cases, and BIRCHFIELD *et al* 1959, 4 cases) This was concluded from measurements of the ventilatory response to one or two gas mixtures containing an increased amount of CO_2 In these studies however, no consideration was given to the possibility that the CO tension measured during air breathing might be situated below the CO threshold (page 51) This fact very likely explains the marked decrease in slope of the response curve during sleep, which the authors mentioned reported On the other hand REED & KELLOGG (1958) (in 3 subjects) and BELLVILLE *et al* (1959) (5 subjects) determining more points on their V—P curves, found a displacement (to the right) of the curve in sleep but no certain change in slope ("sensitivity") The present results are principally in accordance with those of the two latter groups of authors The moderate decrease in slope found in the present series could obviously not have been established from a few cases only

The frequent lack of any noticeable CO_2 drive upon ventilation during (oxygen enriched) air breathing — the 'subthreshold" ventilation — was demonstrable in several subjects also during steady sleep Therefore, the subthreshold drive cannot be ascribed only to the presence of the wakeful state or consciousness as suggested by FINK (1961) In contrast to what here was shown for physiological sleep, the subthreshold drive is absent during general anesthesia (HANKS *et al* 1961) a fact indicating a preservation of certain integrated brain functions in normal sleep

It seems likely that an influence from higher brain centres is partly responsible for the ventilatory drive in the waking state It should be recalled that telencephalic structures including certain cortical areas have been shown to have an influence on respiration (KAADA & JASPER 1952) However, the basic ventilatory drive may as well be due to afferent "collateral" neuronal inflow (GRANIT 1955, ELDRED & FUJIMORI 1958)

It seems indeed possible that the gamma motor system and the afferent pathways from the muscle spindles play an important role for the events during sleep onset Sleep is as is well known also accompanied by a reduction of muscle tone (HOFFMAN *et al* 1956 JOUVET *et al* 1960) This is most likely caused by a loss of the supraspinal drive of the gamma

motor neurons (GRANIT & KAADA 1952 HAMMOND et al 1956 cf GRANIT 1955) Thereby the afferent flow from the muscle spindles must decrease and a secondary reduction of the proprioceptive drive upon the reticular system (including respiratory centres) will then ensue The loop described thus forms a feedback circuit which may participate in sleep onset Probably the proprioceptive system of the respiratory muscles is involved in the events discussed above in a more complex way This question is however at present unclear and it lies mainly outside the scope of the present investigation (cf CAMPBELL & HOWELL 1963)

A decrease in the neuronal stimuli mentioned above may contribute to the change in the CO_2 threshold with a decrease in wakefulness It is however also possible that such factors may affect the slope of the ventilation CO_2 response curve

3 RESPIRATORY STABILITY DURING SLEEP ONSET

A close correlation was found between the displacement during sleep of the CO_2 response curve DsT$_{A\ C}$ and the amount of respiratory oscillation PB during sleep onset Between the subjects the variations of the two factors were large In each individual however the variations were small even on different examinations

The present results do not support the findings of MANGOLD et al (1955) who in 6 fatigued subjects found only an insignificant increase in P$_{CO_2}$ during sleep from high values when awake during the period just preceding sleep In other fatigued subjects who failed to fall asleep the P$_{CO_2}$ was clearly lower One might question whether the subjects who fell asleep represented a selection of stable subjects (page 62) among which continuous sleep pattern in the EEG more rapidly follows the initial signs of sleep

It was shown that not only the amount of ventilatory change but also the change of ventilatory pattern in sleep is characteristic in a given subject Differences in ventilatory pattern were found between the two groups of stability In the 'stable' group the ventilatory frequency was low when the subject was awake and increased during sleep in the 'unstable' group the rate was somewhat higher when awake than when asleep These observations may possibly have a bearing on the recent findings of COHEN & HUGELIN (1961) In nonanesthetized curarized and vagotomized spinal cats they found two different patterns of activity in the phrenic nerve following electrical stimulation of the reticular system (i e arousal) In all cases arousal was accompanied by an increased inspiratory discharge The more common type was characterized by an increased respiratory rate the less frequent one by a reduced rate mainly owing to a prolongation of the expiratory pause In man awake, prominent expiratory pauses often appear during slow breathing

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A decrease in the neuronal stimuli mentioned above may contribute to the change in the CO_2 threshold with a decrease in wakefulness. It is however also possible that such factors may affect the slope of the ventilation-CO_2 response curve.

3 RESPIRATORY STABILITY DURING SLEEP ONSET

A close correlation was found between the displacement during sleep of the CO_2 response curve, $DsT_{A\,C}$, and the amount of respiratory oscillations, PB, during sleep onset. Between the subjects the variations of the two factors were large. In each individual however the variations were small even on different examinations.

The present results do not support the findings of MANGOLD *et al* (1955) who in 6 fatigued subjects found only an insignificant increase in I_{CO} during sleep from high values when awake during the period just preceding sleep. In other fatigued subjects who failed to fall asleep the I_{CO} was clearly lower. One might question whether the subjects who fell asleep represented a selection of "stable" subjects (page 6[illegible]) among which continuous sleep pattern in the EEG more rapidly follows the initial signs of sleep.

It was shown that not only the amount of ventilatory change but also the change of ventilatory pattern in sleep is characteristic in a given subject. Differences in ventilatory pattern were found between the two groups of stability. In the "stable" group the ventilatory frequency was low when the subject was awake and increased during sleep; in the "unstable" group the rate was somewhat higher when awake than when asleep. These observations may possibly have a bearing on the recent findings of COHEN & HUGELIN (1961). In nonanesthetized curarized and vagotomized spinal cats they found two different patterns of activity in the phrenic nerve following electrical stimulation of the reticular system (*i e* arousal). In all cases arousal was accompanied by an increased inspiratory discharge. The more common type was characterized by an increased respiratory rate, the less frequent one by a reduced rate mainly owing to a prolongation of the expiratory pause. In man awake prominent expiratory pauses often appear during slow breathing.

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nes such as during the period of onset of sleep are le s prominent and le s regular and also more tran tory than the respiratory changes Th heart rate was not linked so intim ately to wakefulness function as was the re piratory funct on

During the CO_2 te ts the change in heart rate was very small irrespec tive of the level of wakefulness This al o applies to measurements of arterial blood pres ure These ob ervations may indicate that the type of CO_2 rebreathing in the low concentrations u ed has no marked cardio va cular effect

PERIODIC BREATHING

The results how that periodic breathing (PB) is a normal and common phenomenon during sleep onset During established sleep with a stable EEG pattern PB did not occur It might, however reappear during changes in the depth of sleep

In spite of the fact that periodic breathing during sleep has long been known (BROAD BENT 18 7 Mo o 1886) the phenomenon has not been the subject of much attention MAGNUS EN (1944) however stres ed its o currence during periods of alternating sleep and wakefulness ROBIN et al (1958) also ob erved a PB during sleep but regarded it as a very tran ient phenomenon only present in a few of the r subjects BELLVILLE et al (1959) on the other hand considered a respiratory instability to be a characteristic of the very sl ep function.

The PB during sleep onset often closely resembled the classical CHEYNE STOKES' breathing This type of breathing is commonly considered a sign of a serious organic disturbance of the cardiovascular and/or the central nervous system (cf PRYOR 1951 TALBERT et al 1951) In the present investigation the normal PB however usually showed less regular undula tions in degree of ventilation (V) The phase of hyperpnoea often showed a sudden on et with a maximum occurring within one or two breaths (see Fig 14 s) As a rule the hypoventilation phases did not lead to complete apnoea Furthermore a mean hyperventilation and a mean decrea e in P_{aco_2} have been demonstrated in classical CHEYNE STOKES breathing (BROWN & PLUM 1961 KARP et al 1961) During the normal PB however there was a gradual mean change in respiration in the opposite direction Finally the hypoxic drive on ventilation which plays a prominent part in CHEYNE STOKES respiration proved to be of no essential importance in the PB during sleep on et

The two classical hypotheses the humoral' and the neuronal' of the origin of CHEYNE STOKES' respiration variations in the blood gases (DOUGLAS & HALDANE 1909) or central nervous (supramedullary) regulatory changes (HOFF & BRECKENRIDGE 1954) may be applied in combination in an attempt to explain the occurrence of PB during sleep on et as will be di cu ed below

The two groups of "stability" may possibly be related to the "high" and "low" ventilation groups proposed by SCHAEFER (1958). These groups differed as to respiratory pattern in a manner resembling the "stable" and the "unstable" groups. In contrast to the groups of SCHAEFER no clear differences were found between the present groups concerning the ventilatory response to CO_2 ("CO_2 sensitivity") nor as to the P_{CO_2} during wakefulness at rest. It may, however, be mentioned that the means of P_{ACO_2} in steady state and of SQ in stage A for the "stable" and "unstable" groups both diverged, but only to a small degree, in the same way as in the groups referred to.

A marked variation in SQ and sT_A was found in both groups of stability. There was no apparent correlation between these two values in an individual. The difference in SQ between the two groups was only small. The findings thus suggest that the respiratory "stability" during changes in wakefulness is largely independent of CO_2 sensitivity. It is, therefore, not surprising that no differences in blood pH and standard bicarbonate were found between the two groups.

No difference was found between the two groups of stability regarding the occurrence of "subthreshold" ventilation at rest during wakefulness or during sleep. However, in the most "unstable" cases the "subthreshold" drive markedly decreased during phases of rapid decrease in wakefulness when apnoea or marked hypopnoea occurred. One might question whether this indicates individual differences in the relative reduction of the "non-chemical" (i.e. "subthreshold") drive as compared with the change in "CO_2 sensitivity".

The following explanation of the individual variations in "stability" is suggested. In the "stable" group there is only a small decrease in "CO_2 sensitivity" of the respiratory centres during sleep onset and therefore the degree of respiratory changes as well as that of oscillations (PB) is very small. In the "unstable" group, the change in sensitivity is relatively large, causing marked oscillations and also marked definite changes in respiration with the onset of sleep.

The two stability groups did not differ in pulmonary capacities. Furthermore, neither sleep deprivation nor the time of measurement during the 24 hour cycle had any obvious influence upon the pattern shown by a given individual. It is thus concluded that the degree of respiratory stability is a fundamental quality related to the (autonomic) constitution of the individual.

This conclusion seems to be supported by the observation of an apparently larger tendency to deviation from completely "normal" EEG pattern among the "unstable" subjects as compared with the "stable" ones. Investigations are in progress on this subject (BÜLOW & INGVAR 1963 c).

In many of the present investigations the electrocardiogram (ECG) was recorded simultaneously with EEG and respiration. In this way the variations in *heart rate* were correlated with the variations in level of wakefulness and in the respiratory variables. Also the groups of "stability" and the changes in ventilatory pattern during sleep were here studied for heart rate. The results of this study will be published separately (BÜLOW 1963 b). It was demonstrated that the changes in heart rate during variation in wakeful-

of rapid decrease in wakefulne s is explained by a marked inhibition of the neuronal 'non chemical' drive on ventilation The hypothe is is extended to include al o a concomitant decrea e in sensitivity to CO of the respiratory centres The reduction of ventilation is thus due partly to a transient neuronal inhibition partly to a definite decrease in CO_2 sensitivity At sub threshold CO_2 tensions the degree of reduction in the former drive determines the decrease in ventilation whereas at CO_2 tensions above the threshold the reduction in CO_2 sensitivity is the deci ive factor

2 In the *intermediate phase* of the period of sleep onset a fairly regular periodicity in breathing and in wakefulness appears The present hypothesis explains this fact by the appearence of a more stabilized resetting of the reticular balance mechani ms mentioned in the direction of deac tivation and EEG synchronization The continuation of the PB was observed to be especially favoured by the periodic arousal reactions which in its turn apparently were related to the periodically increased CO tensions the CO_2 arousal thresholds'

When the increased ventilation during arousal lowers the CO_2 tension to levels below the arousal threshold the prevailing tendency to decreased wakefulness can again make itself felt

The sleep inducing mechan sm may perhaps be enhanced by the periodically increased CO_2 stimul in the am manner as during habituat on to monotonou ly repeated stimuli of other or g n

3 In the further course of sleep onset ventilation and wakefulness gradually decrease and P_{ACO_2} and its arousal thresholds' increase In the *final phase* the repeated arousals and the PB disappear with a stabilization of the increased CO_2 tension during sleep

In summary the events in the brain stem (and higher centres) during sleep on et still remain a matter of conjecture The widely accepted concept of the reticular system has however provided a basis for hypotheses in line with those pre ented above

The pre ent investigation is limited to the question how one of the basic functions of the body respiration varies with wakefulness In view of our present knowledge of the reticular system of the brain stem it seems very likely that other functional entities within the body such as the systemic circulation the mu cle tone and the ga trointe tinal function should also show certain functional patterns pertaining to different levels of wakefulness Thi large problem complex which is of primary importance for our under standing of the sleep wakefulne s phenomena will require much work to be clarified There is also evidence suggesting that the muscle tone (the gamma motor activity) varies with wakefulness (von Euler & Soderberg

THE MECHANISM UNDERLYING THE PROCESS OF FALLING ASLEEP, AS INTERPRETED FROM THE CHANGES IN RESPIRATION AND EEG

In the following some further suggestions will be made as to the mechanism of sleep onset

Attempts to describe the neuronal mechanism responsible for the sudden repeated decreases in wakefulness and in ventilation at sleep onset must be purely hypothetical Nothing is known of possible changes during sleep in the physiochemical properties of the nerve cells including the sensitivity of the central or peripheral chemoreceptors to specific stimuli such as CO_2 In a recent thorough survey MORUZZI (1960) points out that the classical views of PAWLOW (1923) of an active internal inhibition and that of MORUZZI & MAGOUN (1949) (cf BREMER 1935 and also KLEITMAN 1939) of a reduced sensory inflow (passive unspecific deafferentation) should both be included in a concept of the production of normal sleep Furthermore recent findings suggest that the inhibitory bulbopontine centres which have an EEG synchronizing effect may quite possibly participate in sleep induction MORUZZI (1960) has also proposed the suprabulbar reciprocal control of the two antagonistic systems of the brain stem — the reticular activating and the synchronizing ones — in order to explain the onset and the progressively increasing depth of sleep The influence of the cortico fugal impulses mediated by the neuronal negative feed back mechanism between the reticular activating system and the cortex should also be taken into account (HUGELIN & BONVALLET 1957) Sleep inducing changes in such reticular mechanisms are very likely favoured by a monotonous non arousing sensory input to which the individual slowly may be habituated The unspecific deafferentation of the reticular activating system would be more subordinate for the onset but essential for the maintenance of sleep

The REM sleep rhombencephalic sleep according to JOUVET (1962) differs principally from the above mentioned sleep mechanisms as it is supposed to involve a different neuronal system REM sleep is assumed to be governed by an active pontine sleep mechanism closely related to the limbic system and responsible for the rapid cortical activity present during this phase of sleep This type of sleep was not observed during periods of sleep onset but only later following periods of ordinary deep sleep

The following *hypothesis* is advanced, explaining the events during sleep onset, in which usually three phases were distinguished

1 Initially, the balance between "activating" and inhibitory" systems in the reticular system of the brain stem changes rapidly and frequently This corresponds to the *introductory phase* (Chapter IV) with its frequent often irregular variations in the EEG level of wakefulness as well as in respiration

The primary cause of this change in balance is at present completely unknown The present results do not support the suggestion by KETY (1961) that an increase of the arterial P_{CO_2} is of fundamental importance for the initiation of normal sleep But the amount of change in respiration during the early phases of decrease in wakefulness was apparently dependent on the prevailing CO tension This was best seen among the unstable subjects and then particularly when the P_{ACO_2} during wakefulness (stage A) was situated clearly below the CO_2 threshold (page 47)

According to the hypothesis the ventilatory change during the phases

5 TENSE ALERTNESS

Some general suggestions will be put forth concerning the cause of the change observed in the ventilation CO_2 response during the state of tense alertness As shown by NIELSEN (1936) and ASMUSSEN & NIELSEN (1957) there is a parallel shift of the response curve to the left (i e a lowering of the CO_2 threshold) during muscular exercise at steady state A change in a similar manner may be assumed for the state of continuous tense alertness (mental work) the large variations in the present observations however preventing definite conclusions During muscular work a neuronal drive on ventilation is of importance (ASMUSSEN & NIELSEN 1949 KAO 1956 for a recent survey see symposium edited by CUNNINGHAM & LLOYD 1963) During tense alertness there may supposedly be an increase in the afferent flow from the muscular proprioceptors as well as in corticofugal volleys the latter mediating facilitating as well as inhibiting effects on ventilation (*cf* MORUZZI 1960) Variations in these two effects were probably the main cause of the substantial ventilatory variations during stage A_a

6 CLINICAL AND PHARMACOLOGICAL ASPECTS

From the clinical viewpoint the establishment of normal reaction patterns of respiration in sleep as well as the statement that a proper CO_2 homeostasis is necessary for stable sleep both have practical implications Conditions characterized by sleep (wakefulness/consciousness) disturbances can be evaluated in respiratory terms So far a few such states have been analyzed

In the three clinical groups examined petit mal epilepsy narcolepsy and the pickwickian syndrome the principal close coupling between EEG and respiration was preserved Each group showed however characteristic deviations from the normal range of variation stated in the present study

The pathological states thus investigated represent defects mainly of the central nervous system It is evident that sleep disturbances due to pulmonary diseases may also be investigated in a similar manner

Some pharmacological investigations have also been performed with the present method An extreme dissociation between level of wakefulness and respiration was demonstrated with a new drug phenoperidine used for neurolept analgesia (BULOW *et al* 1963) In spite of a marked respiratory depression as demonstrated by increased CO_2 threshold wakefulness was preserved behaviourally as well as in the EEG pattern (stage A)

It is apparent from the present study that the changes in respiration

1957) Also the systemic circulation shows certain patterns in sleep (HOFFMAN *et al* 1956, ASAHINA & MATSUI 1962, and others) distinct from those in wakefulness

4 P_{ACO_2} AND THE MAINTENANCE OF SLEEP

Definite sleep never occurred until P_{ACO_2} remained within the limits that existed for *each stage of sleep When during CO_2 rebreathing the* upper limit was exceeded, arousal — or a change in that direction — *occurred At each level of wakefulness this upper limit showed only small* variations The upper limits were therefore considered to implicate "CO_2 *arousal thresholds*", which increased with the depth of sleep

It is concluded that prominent variations in the CO_2 tension of the blood—and therefore also the brain tissue—are not compatible with a stable level of sleep *A proper CO_2 homeostasis is thus a prerequisite for stable normal sleep*

When P_{ACO_2} increased gradually during the CO_2 tests no influence on the EEG level of wakefulness was observed until the "arousal threshold" for the prevailing EEG stage was exceeded At CO_2 tensions below these thresholds, CO_2 rebreathing had, on the contrary, a clear stabilizing effect on any existing PB and also on the concomitant variations in wakefulness The gradual increase in P_{ACO_2}, induced by the CO_2 rebreathing, often proved to enhance the onset of definite, stable sleep This observation may be explained by the fact that each periodic increase in ventilation following an arousal has an ever decreasing influence on P_{ACO_2} when the inspiratory P_{CO_2} is gradually increased

It should be mentioned that PITT *et al* (1907) *observed that CO_2 admixture to inspiratory* air tends to remove the typical signs of CHEYNE STOKES breathing

"RAPID EYE MOVEMENT" SLEEP

During REM sleep the linkage between EEG pattern and degree of ventilation appeared to be less distinct However no clear difference in the linkage as compared with the classical sleep stages could be demonstrated from the present limited number of observations This also holds for the ventilation CO_2 response which roughly corresponded to that during stage B, when the EEG of the REM state and the stage B showed similar amounts of theta activity The possibility of varying cortical influences on respiration during this state, characterized frequently by dreaming should also be taken into consideration

REM sleep has so far not been systematically explored in man (JOUVET *et al* 1960, SCHWARTZ 1962) Its relation to respiration and other basic functions of the body requires further investigation

CHAPTER XII

SUMMARY AND CONCLUSIONS

1 In seventy normal subjects the respiration during wakefulness and sleep was studied Continuous records were made of the EEG the ventilation and the alveolar carbon dioxide tension for periods of three to six hours Various levels of wakefulness were studied including tense alertness wakefulness at rest drowsiness and various levels of sleep

2 Normally there exists a *close linkage between respiration and level of wakefulness* as measured by the EEG This conclusion is based upon more than 1 000 observations A decrease or an increase in wakefulness including tense alertness is accompanied by a change in the corresponding direction in ventilation

The close linkage is present even during sudden or very transient wakefulness changes of a few seconds or less Even such short periods of sleep are generally recognizable on a spirogram from the very onset of sleep

During respiratory steady state the principal difference in degree of ventilation between various levels of wakefulness is preserved

When the level of wakefulness is stable respiration is stable too

The results give further support to the assumption that brain structures regulating wakefulness and respiration are closely interrelated functionally

3 *A change in respiration with wakefulness is accompanied by a change in the ventilatory response to* CO_2 This was found in more than 300 CO_2 rebreathing tests with a technique developed especially for the purpose It allowed repeated measurements of the ventilation P_{ACO_2} response curves with a large number of points for various levels of wakefulness

The decrease in ventilatory response to CO_2 with wakefulness is characterized by an *increased* CO_2 *threshold* A *moderate decrease in slope of the response curve in sleep* is also demonstrated This is interpreted as an indication of a decreased "sensitivity" of the respiratory control system to the CO_2 stimulus The results support the suggestion that the reduced CO_2 response in sleep (the changed setting of the respiratory chemostat) is due both to a decreased "sensitivity" of the respiratory centres to the CO_2 stimulus *per se* and to a decreased neuronal drive on the respiratory centres

with wakefulness should be taken into account in clinical routine examinations of pulmonary functions

In conclusion, a proposal for further clinical research may be made It seems that a continuous analysis of EEG and respiration and probably some other bodily functions, as outlined in the present study, will provide a basis for increased understanding not only of various sleep disturbances but also of mental disorders, characterized by a defective wakefulness regulation In this respect, cases usually diagnosed as "neurosis" are of special interest Many of these show symptoms of tension restlessness (NILSSON 1960) and anxiety in combination with different cardio circulatory, as well as respiratory symptoms (SODERSTROM 1954 BULOW 1961) This view is supported by some preliminary observations with the present technique in patients belonging to the clinical groups mentioned (BULOW & INGVAR 1963 c)

It is stressed that the level of wakefulness should be taken into account in every routine analysis of pulmonary functions

Investigations in progress favour the view that continuous measurement of EEG respiration and probably also some other variables representing basic bodily functions will provide a basis for increased understanding of sleep disturbances but also of various mental (neurotic) syndromes characterized by tension, anxiety or respiratory and cardiocirculatory symptoms The stability ' factor in the respiration wakefulness mechanism may then presumably be used as an index of basic functional properties of an individual

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To my wife Dr KATARINA BÜLOW I am greatly indebted for good help throughout the investigation

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BO ISRAELSSON *Ph D* assisted in the statistical work

The English text was checked by Mr L JAMES BROWN

During wakefulness as well as during sleep, respiration is often governed by factors other than CO_2 as implicated by the presence of a CO_2 *threshold* below which the variations in CO_2 tension has no certain effect on ventilation

The "rapid eye movement" (REM) sleep resembled drowsiness as to ventilation CO_2 response

During tense alertness *there was a moderate mean decrease in CO_2* threshold No certain change in slope was demonstrable

4 The period of *onset of sleep* is characterized by concomitant oscillations in wakefulness and respiration *The degree of periodic breathing* (PB) *is related to the degree of change in ventilation CO_2 response between wakefulness and sleep*

The degree of PB and change in CO_2 response vary widely between the individuals, but are characteristic for the individual Two groups are distinguished, one "*stable*" and one '*unstable*", according to the respiratory stability during changes in wakefulness The former group, with the larger change in CO_2 threshold, shows also a clear decrease in slope of the CO response curve ("CO_2 sensitivity"), in the latter group no clear decrease in slope is demonstrable The results suggest that the changes from wakefulness to sleep in CO_2 threshold and in slope are probably interrelated

The two groups of stability differ in respiratory rate and in the change in this rate between wakefulness and sleep No differences between the two groups were found regarding various other respiratory parameters or age, sex, and body surface area or diurnal rhythm or presence of sleep deprivation

The mechanism of onset of sleep is discussed The initial phase of sleep onset is characterized by rapid and frequent fluctuation presumably in nervous balance mechanisms and also by concomitant changes in "CO sensitivity" of the respiratory centres Definite sleep is accompanied by a stabilization of the change in the setting of the balance mechanisms

5 *Appropriate CO homeostasis is a prerequisite for stable sleep* Stable sleep only appears when the CO_2 tension is increased and then kept within narrow limits This can sometimes be enhanced by CO rebreathing

When the CO_2 tension exceeds an upper limit, an increase in level of wakefulness occurs This upper limit is therefore interpreted as a "CO_2 arousal threshold" It increases with the depth of sleep

6 Some *clinical applications* of the method presented are mentioned In some central nervous or pulmonary mechanical dysfunctions the close respiration wakefulness linkage was preserved though differing in various respects from the normal range stated here

It is stressed that the level of wakefulness should be taken into account in every routine analysis of pulmonary functions

Investigations in progress favour the view that continuous measurement of EEG respiration and probably also some other variables representing basic bodily functions will provide a basis for increased understanding of sleep disturbances but also of various mental (neurotic) syndromes characterized by tension anxiety, or respiratory and cardiocirculatory symptoms The stability ' factor in the respiration wakefulness mechanism may then presumably be used as an index of basic functional properties of an individual

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Subject	Sex	Age	In e tig No	In stig type (se below)	P B	BSA	SQ	$_sT_A$	$D_sT_{A_s}$-A	D_sT_{A} B	D_sT_{A}C	D_sT_{A} D	D_sT_{A} E	SQ D_sT_{A}C	SQ D_sT_{A}D
STABLE GROUP															
V O	f	25	I	D+	0	1 99	0 8	36 5	0 6	1 3^{1}	1	1 8		1 2	1 1
		?5	II	D+	—	1 99	0 7	37 8		0 8^{1}					
		6	III	D+	0	1 99	0 8	43 1^{1}			1 0^{1}			0 8^{1}	
		6	IV	D +	0	1 97	0 7	40 ?	1 0		1 8			1 3	
mean					*0*	*1 99*	*0 8*	*39 4*	*0 8*	*1 1*	*1 1*	*1 8*		*1 1*	*1 1*
K N	f	0	I	D+	(+)	1 6	0 5^{3}	33 2^{2}			2 1^{3}			1 1^{3}	
		0	II	D+	(+)	1 6	0 7	38 3^{1}	1 6^{1}	1 3^{3}	? 1^{1}			1 5^{1}	
		?1	III	D+	+(+)	1 61	0 7	38 4^{4}			1 3	2 0		0 9	1 4
mean					*+*	*1 6*	*0 6*	*36 6*	*1 6*	*1 3*	*1 8*	*? 0*		*1 ?*	*1 4*
D F	m	22	I	(D)s	(+)	06	0 7	38 5							
			II	D	+	2 06	0 8	38 5	1 4		0 0			0 0	
mean					(+)	*2 06*	*0 8*	*38 5*	*1 4*		*0 0*			*0 0*	
M J	f	24	I	D+	(+)	1 77	1 0	40 1	1 4		? 5			2 5	
		24	II	Ds+	—	1 77	0 9	41 ?	0 ?						
mean					(+)	*1 77*	*1 0*	*40 8*	*1 1*		? 5			*2 5*	
I D	f	31		D+	0	1 49	1 2	?9 7		0 8^{1}	1 4	1 6		1 7	1 9
I O	m	27		(D)s	(+)	1 95	0 5	43 7^{4}	1 7		2 3	2 3		1 2	1 2
A A	f	31		D+	(+)	1 55	0 7	36 7		1 0	1 5	1 0		1 1	0 7
I B A	f	36		D+	0	1 98	0 4	38 0	? 6	0 0	0 0	1 0^{1}		0 0	0 4
G H	f	57		D+	0	1 79	0 4	41 1		1 0					
I I	m	48		D+	0	1 0	0 7	36 8		1 2	0 4^{3}			0 3^{3}	
K C	m	40		D+	+	1 95	0 8	43 1		0 7^{3}					
I F	m	6		D+	(+)	1 ?	0 8	41 ?		0 5	1 ?			1 0^{1}	
I A	m	33		D+	+	1 84	0 4	41 8	1 1	0 3	0 9			0 4	
G S	m	18		D	0	1 0	0 7	1? 5		1 3	? 3			1 6	
T S	m	18		D+	0	1 75	1 1	42 ?	1 5	0 0^{1}	0 5			0 6	
L K	m	18		D+	+	1 85	0 6	39 9		1 3	? 5	1 6^{1}		1 5	2 8^{1}
I S	m	?0		D	(+)	1 98	0 4	39 8		1 6^{1}	3			1 3	
V B	m	30		D	0	1 79	0 5	39 ?	0 9	0 0^{1}	0 6^{3}			0 3^{3}	

Subject	Sex	Age	Investig	Investn (see below)	P B	BSA	SO	$_sT_A$	DsT_{1a} A	DsT_A B	DsT_A C	DsT_A D	DsT_A E	SQ DsT_A C	SQ DsT_A D
UNSTABLE GROUP															
B H	m	23	I	D	++(+)	2 14	1 1	33 6			3 3			4 0	
		24	II	D	—	2 14	1 4	36 1	0 0						
		25	III	(D)	+++	2 14	1 0	37 0[4]			2 4	>7 0	>7 0	2 4	>7 0
		26	IV	(N)	++(+)	2 14	0 9	36 3		1 1	2 6			2 3	
mean					++(+)	*2 14*	*1 1*	*35 8*	*0 0*	*1 4*	*2 8*	*>7 0*	*>7 0*	*2 9*	*>7 0*
S B	f	28	I	D+	++	1 74	1 2	36 8		1 5	3 9			4 7	
		29	II	D+	++(+)	1 74	1 1	35 6		2 1	2 8[2]			3 1	
		30	III	Ds+	++	1 74	1 2	38 0		2 0					
mean					++	*1 74*	*1 2*	*36 8*		*1 9*	*3 4*			*3 9*	
B B	m	23	I	D+	++	2 01	0 7	40 4			4 9	4 9		3 4	3 4
		23	II	D+	++	2 04	0 7	40 7		1 3[3]	4 8	4 8	4 8	3 4	3 4
		25	III	Ns+	+(+)	2 01	0 6	41 2	1 4	1 7[3]					
mean					++	*2 04*	*0 7*	*40 8*	*1 4*	*1 5*	*4 9*	*4 9*	*4 8*	*3 4*	*3 4*
M A	f	22	I	(D)s	++	1 70	1 3	40 6		4 7[2]	5 4[2]			7 0[2]	
		22	II	D	++	1 70	0 8	35 9	3 0	3 9	4 6			3 7	
mean					++	*1 70*	*1 1*	*38 3*	*3 0*	*4 3*	*5 0*			*5 4*	
V A	m	47	I	D+	++	1 95	0 6	37 7			3 5	3 3		2 1	2 0
		48	II	D+	++	1 95	0 7	37 7[1]		2 2[1]	4 2[1]	7 9[2]		2 9[1]	5 5[2]
mean					++	*1 95*	*0 7*	*37 7*		*2 2*	*3 9*	*5 6*		*2 5*	*3 8*
K A N	m	27	I	D+	+++	1 97	0 8	40 9			4 8[2]	8 8	8 8	3 8[2]	7 0
		28	II	D+	+++	1 95	0 9	40 7		2 3	4 8[2]	8 8		4 3[3]	7 9
mean					+++	*1 96*	*0 9*	*40 8*		*2 3*	*4 8*	*8 8*	*8 8*	*4 1*	*7 5*
E. N	m	39	I	D+	+++	1 60	1 1	38 5		1 5[3]	4 5			5 0	
		41	II	Ns+	+++	1 60	0 9	37 9		1 9	4 5			4 1	
mean					+++	*1 60*	*1 0*	*38 2*		*1 7*	*4 5*			*4 6*	
K B	f	42	I	D+	+++	1 95	0 6	43 0[4]			3 8	8 2	12 6[1]	2 3	4 9
		42	II	D+	+++	1 95	0 6	44 7		1 8	4 4	8 1[1]		2 6	4 9[1]
mean					+++	*1 95*	*0 6*	*43 9*		*1 8*	*4 1*	*8 2*	*12 6*	*2 5*	*4 9*
I A	f	22	I	D+	++	1 51	1 1	39 8	1 2	1 4		4 2	4 1[2]		4 6
		23	II	Ns+	+(+)	1 51	1 2	37 3	0 6	0 8	2 5	3 7[1]		3 0	4 4
mean					+(+)	*1 51*	*1 2*	*38 6*	*0 9*	*1 1*	*2 5*	*4 0*	*4 1*	*3 0*	*4 5*

Subject	Sex	Age	Investig No	Investig type (see below)	P B	BSA	SQ	sT_A	DsT_A A	DsT_A B	DsT_A C	DsT_A D	$D T_A$ E	SQ DsT_A C	SQ DsT_A D
I J	f	1	I	D+	+ +	1 77	0 6	39 1	0 0	1 9	4 3	7 3		2 6	4 1
		2	II	D+	+ +	1 77	0 7	36 4		1 5	4 0	5 8	5 8	2 8	1 1
mean					+ +	1 77	0 7	37 8	0 0	1 7	4 [illegible]	7 3	5 8	2 7	4 3
A H	f	24	I	(N)	+(+)	1 [illegible]	0 5	35 2[4]		1 4	5 7			2 9	
		24	II	(D)s	—	1 72	0 5	31 6	2 2						
mean					+(+)	1 72	0 5	34 9	2 2	1 4	5 [illegible]			2 9	
S I	m	21	I	(N)	+ +	1 88	0 9	41 5		1 [illegible]	4 5	4 5	4 5	4 1	4 1
		1	II	(D)s	+ +	1 88	1 0	38 4		1 9	4 8			4 8	
mean					+ +	1 88	1 0	40 0		1 6	4 7	4 5	4 5	4 5	4 1
A R	f	5		D+	+(+)	1 53	1 2	37 2		1 7	3 2			3 8	
S S	f	41		D+	+(+)	1 66	1 1	36 1[4]		2 1	5 2			5 7	
J C	m	49		D	+ +(+)	1 99	0 7	33 2	0 0		4 3			3 0	
M L	f	60		D	+ + +	1 52	0 5	40 0		1 [illegible][3]	5 2	10 5[2]		2 6	5 3[2]
F A	m	39		D+	+ + +	1 90	0 5	36 9	2 1	1 9[3]	8 6	8 6		4 3	4 3
H B	f	37		D	+(+)	1 [illegible] 0	0 9	35 7	1 4	1 5[2]					
S L	m	33		D+	+ +	2 01	0 8	36 0		[illegible] 7	4 1[1]			3 3[1]	
G S	m	[illegible]9		D+	+(+)	1 91	0 9	36 8	0 4	0 9[3]	4 9	[illegible]		4 4	6 9
A K	m	26		D+	+ +	1 80	1 0	37 8	0 8	2 6	3 8			3 8	
I B	m	43		D+	+(+)	1 91	1 0	37 0		1 6[1]	2 6	4 6[2]		2 6	4 6[2]
M W	f	[illegible]9		D+	+ +(+)	1 65	1 1	42 8	2 1		3 9	6 0		4 3	6 6
A T	f	5[illegible]		D+	—	1 76	0 5	38 3	0 3	1 7[3]					
A I	f	4[illegible]		D+	+ +(+)	1 65	1 1	37 2		1 5	4 2			4 6	
A C	m	44		D+	+ +(+)	1 75	0 6	37 9	0 6	1 [illegible][1 3]	4 [illegible][1]			2 5[1]	
H F	m	45		D+	+(+)	2 11	0 7	40 0		—					
E H	m	46		D+	+	2 00	0 9	34 7	3 1	3 0					
S I	m	27		D+	+(+)	1 95	0 8	38 3	2 2	0 [illegible][3]	4 [illegible][3]			3 8[1]	
Y T	m	39		D	+ + +	1 [illegible]5	0 7	37 0			6 8			4 8	
I H	m	[illegible]		D	+	1 93	0 7	39 6	1 4	1 5	3 8			2 7	
R T	m	[illegible]		D	+(+)	1 73	0 8	33 8	0 3	2 1	3 3			2 6	
mean						1 82	0 9	37 8	1 4		4 4			3 6	

Explanation of symbols in the table

[1] sT based on only 3 or 4 points
[2] sT based on only 1 or 2 points (near the sV line)
[3] measured during EEG stage B_1
[4] measured during a stage between EEG stage A and B_1
— not possible to estimate (because the subject was awake most of the time)

For classification of degree of PB (periodic breathing) see page 61

D investigated during day time
N investigated during night
Ds slept during next preceeding night
Ns slept during next preceeding day time
( ) not night worker or no night work for several weeks
\+ following one of the above mentioned signs means subjects who were used to night work

3 subjects were not included in the table since the signs of drowsiness and sleep were of too short duration to allow estimation from CO_2 tests of sT_D or sT during sleep

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Contents



Introduction

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The question of absolute sound pressure threshold is barely treated in the literature GRIFFIN (1950) calculated from actual measurements that the minnow had a threshold of response to underwater sounds of about 21-35 decibels (db) above 2 10^{-4} μBar (μB) i e 0 002-0 01 μB and the eel 50-60 db i e 0 06-0 2 μB AUTRUM and POGGENDORF (1951) and POGGENDORF (1952) found sound pressures of 0 003-1 and 0 001-0 01 μB sufficient to elicit response in the catfish (*Ameiurus nebulosus*)

The ranges of sound frequencies that can be perceived are known for a large number of species but information on pitch discrimination is available for only a few Tabl 1 lists these data for several species of fish More extensive reviews of the literature are given by VON FRISCH

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(1938) KLEEREKOPER and CHAGNON (1954) LOWENSTEIN (1957) and SCHWARZKOPFF (1960) With regard to both upper frequency limit perceived and to pitch discrimination Ostariophysi fishes hear much better than nonostariophysids due to the presence in the former of a morphological connection between the swimbladder and the inner ear through the three pairs of Weberian ossicles (VON FRISCH 1936 POGGENDORF 1952) Perception of sound frequencies up to 13 000 cps has been reported for the catfish (STETTER 1929 VON FRISCH 1936) although in most of these species the upper hearing limit lies around 5000–7000 cps

For most nonostariophysids the upper limit is well below 1000 cps Some species however which possess swimbladder extrusions contacting some part of the labyrinth (Sparidae) or air-filled cavities around the inner ear (Mormyridae Anabantidae) are sensitive to tone frequencies up to 1250 and 3000 cps respectively

Pitch discrimination in Ostariophysi was studied by STETTER (1929) who found minnows (*Phoxinus laevis*) able to discriminate two tones one octave apart (100 per cent frequency difference) and in one case even a minor third apart (19 per cent) However these values concern absolute pitch since the experimental fish was not presented with two different tones in immediate succession but had to remember the first tone for some time before the next one sounded WOHLFAHRT (1939) investigated relative pitch discrimination in minnows and found it to be approximately 6 per cent for tones around 1000 cps DIJKGRAAF and VERHEIJEN (1950) improving Wohlfahrt s technique found the threshold for pitch discrimination to be 3 per cent in the 400–800 cps range With the same methods DIJKGRAAF (1952) found the best pitch discrimination for nonostariophysids to be 9 per cent

The basis for pitch discrimination in teleosts is still unknown Their labyrinth has no obvious morphological frequency analysers all three otolith organs are calcite structures embedded in cavities filled with endolymph and covered by membranes which in certain areas are lined with sensory epithelia There would seem to be two possible mechanisms upon which pitch discrimination can rest in the absence of a morphological discriminator namely 1) synchronization between sound frequency and frequency of impulse discharge and 2) use of different sensory units for different sound frequency ranges

Electrophysiological data on the sense of hearing in teleosts are scarce ADRIAN CRAIK and STURDY (1938) obtained microphonic responses from the eel but no action potentials ZOTTERMAN (1943) succeeded in

Table I

Upper hearing limit and pitch discrimination in some teleost fishes

Family	Species	Upper frequency limit of hearing (cps)	frequency discrimination for given pitch (cps)	Author
OSTARIOPHYSI				
Cyprinidae	*Phoxinus laevis*	5000–7000	19 6 1000 3 400–800	STETTER 1929 WOHLFAHRT 1939 DIJKGRAAF and VERHEIJEN 1950
	Carassius auratus	3480		STETTER 1929
Characinida	3 species	6960		v. BOUTEVILLE 1935
Siluridae	*Amiurus nebulosus*	13 000		STETTER 1929 v. FRISCH 1938
NON-OSTARIOPHYSI				
Anguillidae	*Anguilla anguilla*	488–650	100	DIESSELHORST 1938
Gobiidae	*Gobius paganellus*	600–800		DIJKGRAAF 1949
	G. niger	800	9 150 12 300	DIJKGRAAF 1952
Sciaenidae	*Corvina nigra*	1000	12 395	———
Sparidae	*Sargus annularis*	1250	under 9 150 15 % 300–450 over 30 600	———
Mormyridae	*Gnathonemus sp*	2794–3136	12	STIPETIC 1939
	Marcusenius isidori	2079–3100	25	DIESSELHORST 1938
Anabantidae (Labyrinthici)	6 species	2637–4699		SCHNEIDER 1942

recording conducted impulses from the saccular nerve of isolated labyrinths of burbot (*Lota vulgaris*) and pike (*Esox lucius*) but made no detailed study of the nerve response

The aim of the present investigation was to elucidate the mechanism of pitch discrimination by recording the electrical activity of single neurons of the acoustic nerve and to study the response to pure tone

(1938), KLEEREKOPER and CHAGNON (1954) LOWENSTEIN (1957) and SCHWARZKOPFF (1960) With regard to both upper frequency limit perceived and to pitch discrimination Ostariophysi fishes hear much better than nonostariophysids due to the presence in the former of a morphological connection between the swimbladder and the inner ear through the three pairs of Weberian ossicles (VON FRISCH 1936 POGGENDORF 1952) Perception of sound frequencies up to 13 000 cps has been reported for the catfish (STETTER 1929 VON FRISCH 1936) although in most of these species the upper hearing limit lies around 5000–7000 cps

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In preliminary experiments some 30 isolated labyrinths of sculpin codfish goby perch pike eel catfish and roach were also prepared, but except for two of pike did not survive Such preparations have the advantage that the different branches of the acoustic nerve can be followed visually to where they spread out over the sensory epithelia whereas in intact preparations with many species it is difficult to determine even the major subdivision of the acoustic nerve into which the electrode has been put

The general pattern of response to sound stimulation could be studied in isolated preparations but because the sound conducting system i e the skull is gravely interfered with results concerning the quantitative relation between sound frequency and response would hardly be trustworthy For this purpose the least possible interference with the fish is required In addition to those with the sculpin therefore experiments with 40 intact preparations of the other species mentioned above were performed but results were not satisfactory and will not be included in this paper

FISH HANDLING AND OPERATION The fish was initially anaesthetized by being placed for a few minutes in a solution of 1 part ethyl m aminobenzoate (MS 222 Sandoz Ltd Basle) in 5000 parts sea water and then was clamped in a holder (Fig 2) consisting of two parallel rectangular perspex pieces (length 20 cm) separated by a stainless steel rod A number of thin steel rods mounted opposite each other through holes in the perspex pieces clamped the fish securely at the base of the dorsal fin and at intervals along the trunk An additional pair of adjustable rods held the fish above the eyes and kept its head in place An alternate arrangement was also used in which the eye bars were released and the fish s head held loosely in place with rubber bands attached to a screw in the nasal bone Although the general pattern of response was the same with both arrangements the head clamp did *influence* skull vibration Therefore results pertaining to the relation between sound frequency and response are based exclusively on data from 15 experiments with the rubber band arrangement

The holder was fixed so that only the dorsal part of the skull trunk and dorsal fin was above the water surface The holder itself was out of water (except for the clamping bar tips) to prevent undesirable stimulation by transmission of vibration from the water through the holder into the fish The water temperature was kept at 10–14 C

stimulation in general and to different tone frequencies in *particular* Experiments were performed with intact specimens of a nonostariophysid fish the sculpin (*Cottus scorpius*) Similar experiments on Ostariophysi species were not successful

This paper will describe first the types of spontaneous activity encountered and then the general pattern of response to acoustic stimulation concluding with a more specific analysis of the relationships between response and sound intensity and frequency

Materials and Methods

FISH The sculpin *Cottus scorpius* was chosen as the experimental animal because of its broad head and well-branched acoustic nerve the *utricular saccular and lagenar divisions of which were all easily accessible* with microelectrodes (Fig 1) Forty-three adult fishes between 18 and 25 cm long were used

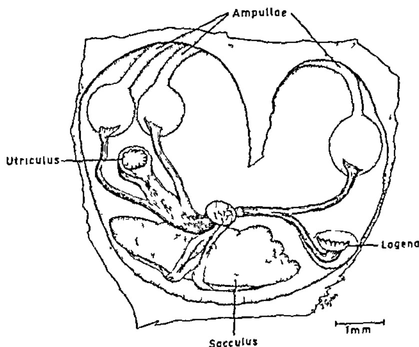


Fig 1 Dorsomedial view of right labyrinth of *C ttus scorpius* Brain and left part of skull removed

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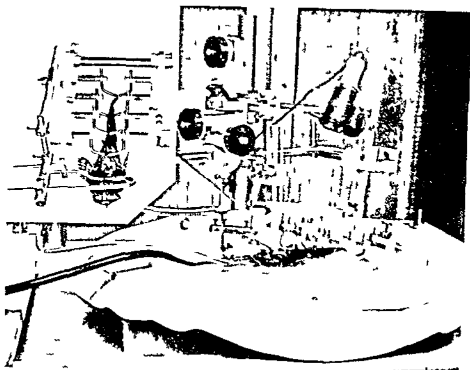


Fig 2 Fish holder and experimental set-up The fish is somewhat elevated for photographic purposes Fish holder and micromanipulator are fixed to steel rods supported by a rectangular iron frame resting on the experimental table A glass capillary *microelectrode is seen lowered into the acoustic nerve by means of the* micromanipulator The thick black cable leads to the hydrophone seen near the fish s head

With the fish under MS-222 narcosis the left caudal part of the brain and the left trigeminal vagal and stato-acoustic nerve roots were exposed Before the recording the fish was immobilized by an intramuscular injection of 0 2–0 3 mg tubocurarine chloride (Nygaard & Co Oslo) per 100 g body weight The gills were continuously supplied with running water through plastic tubing from an elevated vessel

THE EXPERIMENTAL TANK was made of soft plastic sheeting (thickness 0 5 mm) shaped much like a hat (upside down) with top and bottom diameters *30 and 23 cm respectively* and height 15 cm At the top the plastic extended outwards to a circular 15-cm-broad rim nailed to a plywood ring which in turn rested on a tyre inner tube and foam plastic The hat s crown passed *through a hole in the experimental* table and the bottom also rested on foam plastic and inner tubing The loud-

speaker was mounted to a central opening in the bottom The designing of a suitable tank constituted one of the main technical difficulties and several types were tried with the arrangement outlined above no vibrations (as measured by a vibration pick up Bruel and Kjær Copenhagen) were transmitted from the tank to the fish holder or electrode-holder and no pronounced resonance frequencies were found in the range studied A slight modification of this set up equally satisfactory was also used namely an elongated container (60 × 30 × 12 cm) of soft plastic sheeting welded to an air filled plastic tube

SOUND STIMULATION AND RECORDING The loudspeaker was a Philips EL 7030/01 10 W with watertight diaphragm (diameter 6 cm) Only pure tone stimulation was applied delivered by an RC-generator (Philips GM 2317) and an audio power amplifier (either one locally built or a Quad II Huntingdon England) No measurements of distortion or harmonic content were made sound waves recorded by the hydrophone were judged as being sinusoidal from their appearance on an oscilloscope The output from the loudspeaker was frequency-dependent as seen from Fig 3 which shows sound pressures measured at different frequencies for three fixed a c voltage input values

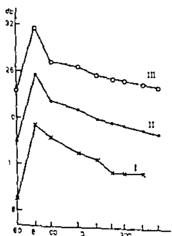


Fig 3 Relation between sound pressure and frequency for constant input voltage to the power amplifier Curves I II and III represent relative input voltages of 1 2 and 4 respectively Sound pressures in this and subsequent figures are given in db above 1 µB

Sound pressures were measured in absolute values by a calibrated barium titanate transducer (Hydrophone Model LC-34 Atlantic Research Corp Alexandria Va) with linear response characteristics in the 10–15 000-cps-range and a sensitivity of −95 decibels (db) referred to 1V/dyne/cm (1 V/μ Bar) Signals from the hydrophone were fed into an a c cathode follower with effective input impedance of over 500 M Ω and a tested gain of 0 9 from 20 to 20 000 cps

Sound pressures in this paper are given in db above 1 μBar a common reference level for underwater sounds (KINSLER and FREY 1950 pp 435–436) and were measured with the hydrophone placed horizontally just below the water surface in the place of the fish s head For any given frequency sound pressures did not vary more than 3 db when measured horizontally within and in the immediate vicinity of the space occupied by the rostral two-thirds of the fish during the experiment Vertically however variations in sound pressures were around 6 db/cm just below the surface and roughly 3 db/cm at a depth of 3 cm The pressure gradient experienced by the fish was thus probably 10–12 db from its ventral side to the water surface which was 2–3 mm below the top of the skull However the neurocranium experienced a pressure gradient of around 6 db as it is only 1 5 cm high

The background noise level in the water amounted to 1 μB (= 0 db) for frequencies above 100 cps and could for lower frequencies be 1–2 db higher

Sound stimuli were delivered usually as tone pips of 1–2 sec duration The shortest intervals between pips were 5–10 sec during which frequency and/or intensity were changed Standard frequencies used were 60 80 100 150 200 250 300 400 and 500 cps each one delivered at as many intensities and as many times as possible until the neuron was lost The 100–500-cps- (or 500–100-) range was tested first the 60- and 80-cps-tones second *since the lower frequencies were more apt to* destroy the neuron To neurons which responded for sufficiently long periods sound stimuli of up to one minute s duration were also delivered

RECORDING OF NERVOUS ACTIVITY Experiments lasted from one to six hours during which only from one to six successful recordings could be obtained Microelectrodes were used for the recording of single unit activity in the utricular saccular and lagenar branches of the acoustic nerve They either were micropipettes (filled with 3M KCl) or occasionally were made of tungsten (HUBEL 1957) diameters were less than

1 μ and 1-2 μ respectively Action potentials were recorded singlesidedly or differentially (with the indifferent electrode on the medulla oblongata) through a d c cathode follower input stage with input impedance greater than 200 MΩ and grid current less than 10^{-11} A Frequency response was limited by the resistance (10-30 MΩ) and capacitance (not measured) of the microelectrodes not by the amplifying system The preparation was grounded through the stainless steel eye bars or the dorsal fin bars

Nerve action potentials and signals from the hydrophone were amplified by two d c amplifiers (251 A American Electronics Lab) displayed simultaneously on a chopped beam oscilloscope (Tektronix 532) and photographed on running film (Grass Kymograph Camera) For recording of nervous activity the amplifier was usually RC-coupled with a time constant of 0 001 – 1 sec In later stages of the work the amplifiers and the chopped beam oscilloscope were substituted by a dual-beam high gain oscilloscope (Tektronix 502)

Some of these experiments were also recorded on a two-channel tape recorder (Tandberg Model 6 Oslo) and later (if necessary) played back on the oscilloscope for photographing The resulting distortion of the action potentials was unimportant for the determination of discharge rates

Results

I General

On introduction of the microelectrode into the acoustic nerve electrical activity was usually obtained from single neurons lasting from a few seconds to more than an hour A large proportion of the single units showed spontaneous discharge On the basis of the general appearance of this discharge the neural units could be classified into three groups those with irregular those with regular and those with burst activity A fourth type of unit showed no activity (Fig 4)

More than half of the units responded to pure tone stimulation The response consisted of the initiation of action potentials in units without spontaneous activity (Fig 4 *D*) and of an increase in discharge rate in units with irregular (Fig 4 4 8 *C D*) or regular spontaneous activity In units with spontaneous burst activity sound stimulation caused a disruption of this activity into a regular discharge of the same frequency as the stimulus tone (Fig 4 *B* 8 4 *B*)

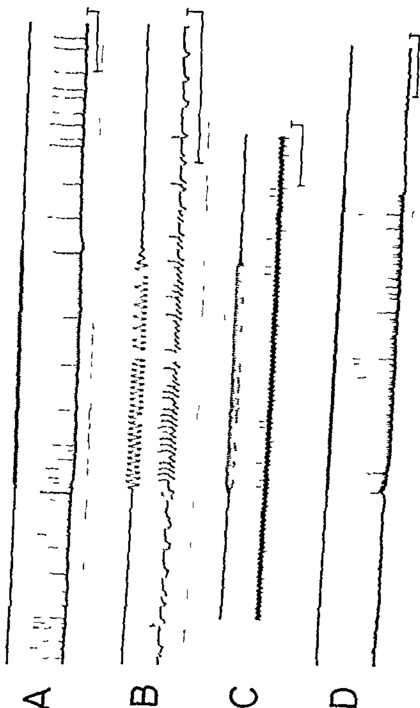


Fig 4 Spontaneous activity records of the four main types of units in the acousti nerve and their responses to a 100-cps tone 28 db above 1 μB Vertical bar 2mV horizontal bar 250 msec Top tracing in each record is signal from hydroph ne Upward deflections represent positive voltages in this and following records *A* Irregular spontaneous activity averaging 20–30 spikes/sec lagenar branch *B* Spontaneous burst activity utricular branch Record interrupted for 1 se *C* Regular spontaneous activity utricular branch No response

Occasionally activity was obtained from a number of units simultaneously The spikes were negative and of low amplitudes in such records and a detailed analysis of the response could not be made

Action potentials from single units were usually monophasic positive spikes but occasionally were positive negative spikes with the positive phase the highest The criteria for identifying a particular discharge as being recorded from a single unit have been enumerated by several investigators (ADRIAN and BRONK 1928 BROOKHART MORUZZI and SNIDER 1950 AMASSIAN 1953 MOUNTCASTLE, DAVIES and BERMAN 1957) The most important features are that spikes are alike in appearance have constant amplitudes bear an all- or- none relationship to stimulus intensity and disappear immediately upon a slight movement of the recording electrode In the present experiments amplitudes were from less than 1 to 12 mV and durations were from 1 to 2 msec The exact time courses of the rising and falling phases cannot be given due to capacitative shunting to ground which varied with depth of electrode and amount of spinal fluid covering the brain Significant abrupt d c potentials were not recorded before the appearance of single unit activity A shoulder on the rising phase of the action potential was frequently seen and sometimes the rising phase did not exceed the shoulder (Fig 5) probably indicating a conduction block at the recording locus These two phenomena indicate that the action potentials were recorded extracellularly and were probably picked up at or close to the somata of the bipolar neurons of the acoustic nerve

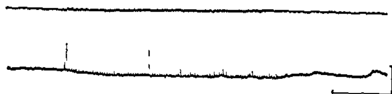


Fig 5 Single unit response to a 100-cps tone Half-size action potentials probably indicate conduction block at the recording site Vertical bar 4 mV time bar 250 msec

The perikarya of the neurons in the acoustic nerve of *[illegible]* are 15–30 μ in diameter (KRISTOFFERSEN and ENGER in preparation) Therefore the continuous registration of electrical activity for more than an *hour* seems inconsistent with intra cellular recording (considering the experimental set-up) as do also the low amplitude action potentials Furthermore two units both with positive spikes were

occasionally observed simultaneously indicating that the recording was extra cellular Lowering or heightening the electrode did not – except in a few cases – change the polarity of the action potentials whereas the amplitude changed from several millivolts to the microvolt level Returning the electrode to the previous position could often bring the spikes back again Obviously in such cases the electrode passed the neuron while in those instances where the neuron lay under neath the electrode tip an electrode lowering caused injury discharge

Visual inspection can determine only in which of the three nerve branches *utricular saccular or lagenar* the electrode was placed Upon penetration of the nerve with the electrode activity was successively obtained from a few units only and sometimes from none at all Histologically the acoustic nerve is composed of bipolar neurons whose cell bodies are scattered throughout the nerve not clustered into definite nuclei (KRISTOFFERSEN and ENGER in preparation) If it is assumed therefore that the electrode picked up spikes from cell bodies only one should expect to get no more than a few units on record each time the electrode was slowly and carefully pushed through the nerve

Positive and positive-negative extracellular spikes have been reported by a number of authors and there seems to be general agreement that they are picked up at or very close to active neurons GRANIT and PHILLIPS (1956) and PHILLIPS (1959) reached this conclusion from experiments with Purkinje cells of the cerebellum and Betz cells of the cerebrum of cats respectively as did LI CULLEN and JASPER (1956) and MOUNTCASTLE DAVIES and BERMAN (1957) from microelectrode studies of somatosensory cortical potentials and TASAKI POLLEY and ORREGO (1954) and FREYGANG (1958) from investigation of single neurons in the lateral geniculate nucleus On the other hand TASAKI (1954) using ultrafine glass micro pipettes recorded activity in single auditory nerve fibres of guinea pigs and got positive action potentials both before and after the appearance of a resting potential KATSUKI et al (1958) reported the same from experiments with cats These authors assumed the electrode tip to have been situated in the myelin sheath and this may have also happened in the present experiments as in *Cottus* the cell bodies seem to have a thin myelin sheath In this investigation however the question of exact recording site constitutes a minor problem and will not be considered further

Injury potentials were frequently encountered and were recognized by an initial high-frequency train of impulses which quickly decreased both in frequency and amplitude and abruptly stopped Such potentials appeared both during movement of the electrode with the micromanipulator and during actual recording from single units In the latter case they were probably caused by the electrode penetrating a cell as a result of a slight movement of the fish Direct current recordings showed that injury potentials were usually preceded by a d c shift of more than 10 mV which probably indicated penetration of the cell membrane by the recording electrode

Units which were systematically investigated did not react to the

electrode at all or gave at most a short burst of impulses with no immediate decrease in spike voltage These impulses were probably initiated mechanically as was also reported by ALANIS and MATTHEWS (1952) from ventral horn cells of the frog There was no indication that such mechanical factors had any prolonged influence on the spontaneous activity Changes from one type of activity to another or long-term changes in average values of discharge rates were not observed

II Spontaneous activity

As described above the spontaneous activity demonstrated by the majority of units was of three types irregular activity burst activity and regular activity Fig 6 shows a histogram analysis of spike intervals typical for each type

Irregular activity (Fig 4 *A* 8 *C* *D* 12 *A*) was characterized by average discharge rates of from fewer than 5 to 80 spikes/sec depending on the unit Fig 6 *A* shows the spike interval distribution of one such unit the intervals are irregularly scattered and there are no obvious preferred interval durations

Occasionally the mean interval frequency of a unit could fluctuate but the causes for this were not systematically studied. Sometimes fluctuations seemed to be brought about by opercular movements when these were large as during light anaesthesia This would not necessarily mean that activity was caused by me ha nical stimulation (such as movement of the electrode against the cell membrane) but rather that the water could produce sound by flowing over the gills Such fluctuations were namely greatly enhanced when air bubbles had entered the mouth cavity through the plastic tubing Even the smallest respiratory movements would then cause marked bursts of impulses which were never observed when recording from the ampullae of the semicircular canals or the lateral line nerve

Irregular spontaneous activity was the type most frequently encountered In the utricular and lagenar nerves about half of the units showed activity of this type and in the saccular nerve about one-third (Table II) but the difference is not statistically significant

In units with low sound thresholds at least part of the irregular spontaneous activity was apparently not spontaneous but represented response to low frequency background noise (see below)

Burst activity was characterized by a grouping of impulse discharge into bursts of generally 2–4 impulses (Fig 4 *B* 8 *A* *B* 13) the number varying even for the same unit with numerous single discharges between bursts Each burst lasted up to 20 msec and burst frequency lay

within 20–70/sec The total discharge frequency never went below 50 or exceeded 200 action potentials/sec The spike frequency in individual bursts was 300–400/sec In rare cases up to 10 impulses per burst were recorded with a correspondingly longer burst duration of 25 msec

Fig 6 *B* illustrates how *the spike intervals of a unit with burst activity* occur in two definite modes the one around 3 msec corresponding to the spike intervals within each burst and the other around 10 msec corresponding to a mixture of intervals between bursts and between bursts and single spikes

Regular activity was characterized by a regular discharge at rates of 15–60 spikes/sec (Fig 4 C) Fig 6 *C* shows a typical interval-distribution of a unit whose mean discharge frequency was 58 spikes/sec Twelve per cent of the units studied showed regular spontaneous activity and were slightly more common in the utricular than in the saccular and lagenar nerves but this difference was not statistically significant No specific statistical criteria were necessary to distinguish regular from irregular activity because they were always so clearly differentiated

Table II summarizes the distribution of units with the three types of spontaneous activity and without spontaneous activity in the utricular saccular and lagenar nerves Only those units were tabulated for which *it was possible to obtain satisfactory records of a reasonable duration* both of spontaneous activity and sound response Short-lasting units which simply have been classified as sound-responsive or non-responsive are not included which may introduce a selection bias in favour of larger neurons It should be noted however that the recording conditions were the same *in the three nerve branches as they all ran horizon*tally in the region of recording and that the somata of the neurons have diameters of 15–30 μ and are scattered throughout the nerves For units not showing spontaneous activity there is also a possibility of a systematic error in that their number may be under-represented compared with units showing *spontaneous activity It is easier to pass a silent* neuron than one with spontaneous discharge and silent neurons which do not respond to sound will of course not be registered Except for these limitations however the distribution of neuron types given in Table II is probably fairly representative of the acoustic system of the fish

The probability that this entire distribution is random as determined by the χ -test is 0 02 From the individual probability values given in

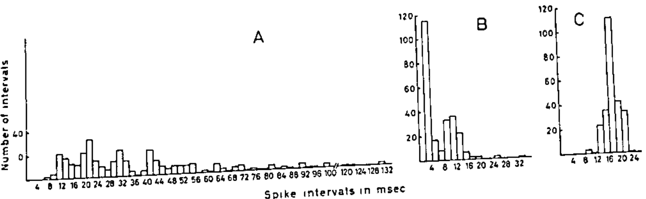


Fig 6 Spike interval distribution of th three types of spontaneous activity *A* Irregular activity averaging 28 spikes/sec *B* Burst activity with 48 bursts/sec 2 4 spikes/burst and an average of 145 spikes/sec *C* Regular activity with 58 spikes/sec

Table II

Distribution of neurons classified by type of spontaneous activity in the three branches of the acoustic nerve

Nerve branch	Utricular		Saccular		Lagenar		Probability for random distribution (P)
No. of units	Total 22	(Sound-responsive) (13)	Total 50	(Sound-responsive) (46)	Total 52	(Sound responsive) (42)	
Irregular activity	11	(6)	17	(16)	27	(23)	0.3
Burst activity	5	(5)	12	(12)	2	(2)	0.02
Regular activity	4	(0)	4	(1)	7	(1)	0.5
No activity	2	(2)	17	(17)	16	(16)	0.2

the table for the even distribution of activity types it can be concluded that units with regular and irregular activity, as well as units without spontaneous discharge may well be evenly distributed among the three nerve branches while units with burst activity are probably rare in the lagenar as compared with the utricular and saccular branches.

III. *Response to sound stimulation*

A. GENERAL

Over half of the units obtained responded to pure tone stimulation as shown in Table II. In a final series of experiments on 12 fishes activity was obtained from 33 utricular, 37 saccular and 37 lagenar units and respectively 12, 35 and 23 (36, 95 and 62 per cent) responded to acoustic stimulation. Each time a unit was obtained it was immediately tested with a 100-cps tone at a moderate sound pressure and then at successively higher intensities if no response was recorded. Unfortunately many of these units were shortlasting, were not classified with respect to type of spontaneous activity and thus were not included in Table II. However all units which lasted for at least 10 sec were included in the second calculations and the proportion of sound responsive units did not deviate significantly from those given in Table II.

The great majority of the saccular units responded to sound whereas only one-third and two-thirds of the utricular and lagenar units did so. This difference between the saccular and the two other receptor areas is statistically significant at the 1 per cent level while the difference

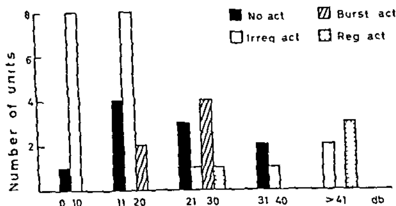


Fig 7 Frequency and type of nerve unit in different sound threshold ranges Stimulus tone 100 cps Units with thresholds above 41 db were non responsive for sound pressures up to 50 db

between utricular and lagenar units is not as tested by the χ^2-test Units with spontaneous burst activity never failed to respond to sound (units without spontaneous activity are of course in the same class) Irregular activity units were partly responsive partly non-responsive to sound stimulation and regular activity units responded only to low frequency high-intensity tones if at all

The sound threshold defined here as the minimum sound pressure necessary to change the firing pattern of a unit was determined either directly or by extrapolation from suprathreshold response values The latter procedure was used only when the lowest sound pressure delivered elicited a weak response (as in the unit plotted in Fig 17 *a* p 37) Fig 7 shows the four types of units arranged according to thresholds for a 100-cps tone (data from experiments where the fish s head was held by rubber bands) There was great variation even within types Units with irregular spontaneous activity covered the entire sound pressure range but those among them which were sound responsive (threshold below 40 db) had on the average a lower threshold (12 db) than any other type of unit Individual units without spontaneous activity could have low thresholds but the average value was the same as for burst activity units (21 db) Units with regular spontaneous activity were almost always non responsive Since the highest sound pressure delivered was 50 db above 1 μB it is possible that units listed as non-responsive might have been responsive for still higher intensities

Only units which gave response to sound stimulation will from now on be considered Those regular-activity units which responded to sound stimulation behaved like high-threshold-responsive irregular activity units and will not be dealt with separately

B PATTERN OF RESPONSE

The response to pure tone stimulation shown in Fig 4 8 9 12 and 13 was of two patterns *the adaptive and the non-adaptive In the first* the response consisted of an increase in rate of discharge which was highest during the initial phase of stimulation and gradually decreased or adapted (Fig 4 *D*, 8 *C D*, 9 *A*, 10 *C–G*) The initial high firing rate was of the same frequency as the stimulus tone (here called *following response*) or a *multiple thereof* The adaptation occurred in one or two steps a fast one completed within 200 msec (Fig 10 *C–F*) and a slow one if present lasting even through the longest stimulation periods tried namely 1 minute (Fig 11) The slow adaptation was present only when the firing rate was considerably above the spontaneous level As the action potentials had a definite phase relation to the sound waves (easily seen by comparison with the sine-wave recording from the hydrophone) adaptation did not involve a smooth increase in time intervals between successive spikes but rather the dropping out of an increasing number of spikes from the following response pattern The adaptive response was found in all units except those with spontaneous burst activity

The second type of response pattern – the non-adaptive – was found in neurons with spontaneous burst activity only Sinusoidal sound waves disrupted this activity into the following response (in some cases double following) and there was little or no adaptation (Fig 8 *A B* 10 *A B* 13)

Units with irregular spontaneous firing rates above 50–60/sec might show weak adaptation simply because the level of spontaneous activity was high In one case a unit with spontaneous burst activity showed moderate adaptation This unit had only 2 or 3 spikes per burst and under 20 bursts/sec which is *fewer than any other unit with spontaneous* burst activity

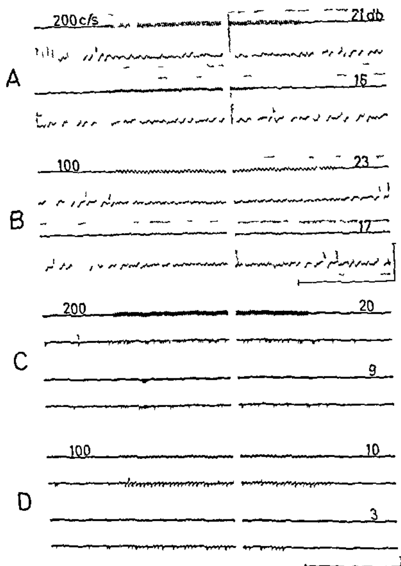


Fig. 8 Response to acoustic stimulation of two sensory units *A B* non adaptive response sacculus *C D* adaptive response utriculus Sound frequency and pressure are given on the left and right respectively of each record Vertical bar 4 *mV* time bar 250 msec Records cut for 1 sec (*A* top *B* bottom) or 0 5 sec (all others)

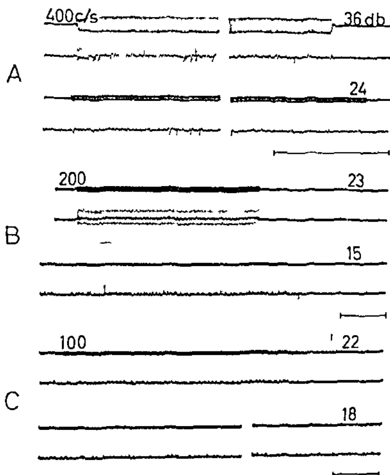


Fig 9 Adaptive responses in a unit whose discharge rate did not exceed the sound frequency utricular nerve branch Records played back from a tape recorder therefore no voltage calibration given Time bar 250 msec Record A cut for 1 sec C (bottom) for 0 5 sec

C EFFECTS OF PREVIOUS STIMULATION ON THE RESPONSE AND POST-STIMULATORY DEPRESSION OF SPONTANEOUS ACTIVITY

The usual stimulus duration of 1–2 seconds elicited the same response to each of two successive stimuli separated in time by at least a couple of seconds A longer lasting stimulus however caused a definite reduction (measured as impulses/sec) in the response to a second stimulus An example is shown in Fig 11 where an irregular-activity unit was stimulated with a 100-cps tone for 60 sec It can be seen that the slow adaptation lasted throughout the stimulation period After the end of this initial

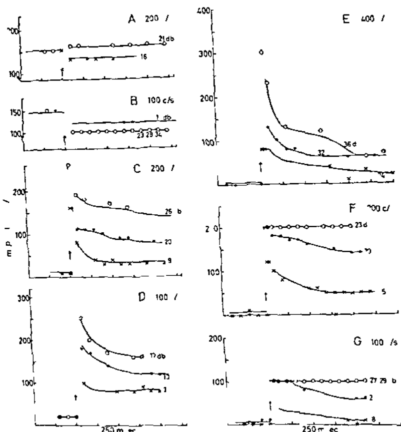


Fig 10 Adaptation rates and relation between response and sound intensity A B a non adaptive unit C D and E F G two adaptive units Most points represent no impulses per 100 msec times 10 Dotted lines indicate that points represent no impulses per 25 or 50 msec times 40 or 20 Arrows indicate start of stimulation Responses are of units represented in Fig 8 and 9

and intensity were delivered The initial phase (first 100 msec) of the response to short pips reached its normal state in about 15 sec while the adapted phase required about 60 sec to reach its stable level (compare with the first part of the response to the 60-sec stimulus in Fig 11) There was a striking correspondence between the recovery time for the full-sized normal response and the recovery time for the spontaneous activity The latter always showed a post stimulatory depression (Fig 4 A B) as also reported by LOWENSTEIN and ROBERTS (1951) for elasmobranchs

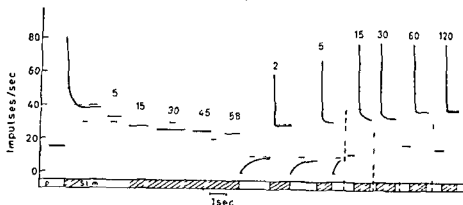


Fig 11 Response to a 60-sec stimulus tone (100 cps) and subsequent responses to 1-sec tone pips of the same frequency and intensity Lagenar unit Numbers on graph give time in seconds after start of stimulation (left half of figure) and after end of the 60-sec stimulation period (right half) Vertical broken lines (right half of figure) indicate breaks in record Stimulation periods indicated by hatched areas at the bottom

D RELATION BETWEEN SOUND INTENSITY AND RESPONSE

In adaptive units the threshold response consisted of a slight initial increase in firing rate (units with spontaneous activity) or a few action potentials during the first phase of stimulation (units without spontaneous activity) As sound pressure increased the initial phase of the response had a few impulses and upon further increase a train of impulses at or above following frequency At higher pressures sound frequencies up to *150* cps could give following response for more than *1* second whereas 300 cps and above never elicited more than a few spikes at following rate

Two types of adaptive units could be distinguished 1) those where the initial phase had *2–3* spikes per sine-wave for frequencies of *100–200* cps at high intensities such units always showing higher initial than steady discharge rates (Fig *8 D 10 C D*) and 2) those where the discharge rate never exceeded the frequency of the stimulating sound As intensity increased these latter units failed to show adaptation but prolonged their initial phase of following response and at sufficiently high pressures reached a plateau of steady following discharge (Fig 9 10 *E–G*) indicating that the adaptation is probably not a matter of nerve fibre accommodation but of the mechanical properties of the sensory cells

The relation between discharge rates and the logarithm of the sound

pressure was approximately linear over a considerable range of intensities to which a unit responded with the curve often flattening out at the highest and lowest intensities (Fig 17 p 37) As intensity increased the spike frequency finally reached a level above which further increase in sound pressure had no effect – at least not up to 50 db above 1 μB which was maximum for sinusoidal output from the loudspeaker This maximal response was ordinarily reached at sound pressures 10–30 db above threshold. For frequencies of 300 cps and above maximal response has never been reached as the output from the loudspeaker was insufficient

In *the non adaptive units* the threshold was determined as the minimum sound pressure disrupting the spontaneous burst activity At low intensities this happened for short intermittent periods in which trains of spikes at following frequency were seen then as intensity increased to about 10 db above threshold the following response became complete (Fig 8 *A B* 10 *A B*) Even a 20 db increase above threshold the highest tried had no further effect on the response Occasionally burst activity units showed some double following response (Fig 4 *B*) but this occurred at various sound pressures and was not a result of high intensity stimulation

E. RELATION BETWEEN SOUND FREQUENCY AND RESPONSE

Sound responsive units in all three nerve branches responded to pure tone stimulation of low frequencies but as frequency increased three different types of units were clearly distinguishable namely 1) units not showing spontaneous activity which responded only up to about 200 cps (Fig 12 *B*) 2) units with irregular spontaneous activity which responded to frequencies up to 300–500 cps (Fig 12 *A*) and exceptionally 700 cps and 3) spontaneous-burst activity units which showed following response up to 200–300 cps (Fig 13 *B*) In Fig 14 the firing rate during the adapted state of the two units shown in Fig 12 is plotted in relation to the sound frequency Plotting of the initial response showed the same relationship but displaced to higher levels of discharge rates Exactly equal sound pressures were not delivered for all frequencies for each unit but by rough interpolation a curve could be drawn for responses expected to tones 30 db above reference level There was a clear distinction between the two units While one was sharply refractive to frequencies above 200 cps the other responded to all frequencies

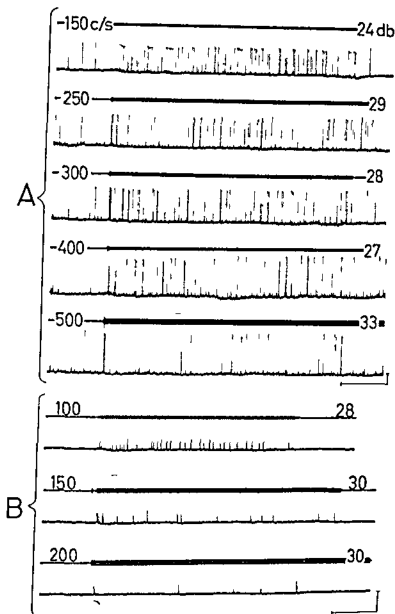


Fig 12 Relation between sound frequency and response A Lagenar unit with irregular spontaneous activity averaging 20–30 spikes/sec B Saccular unit without spontaneous activity Vertical bar 4 mV time bar 250 msec

up to 500 cps For a further analysis of this phenomenon two parameters have to be considered namely the *threshold sound pressure* and the *sensitivity*

The *threshold* intensity varied considerably for different units as already shown in Fig 7 but for units with the same type of spontaneous

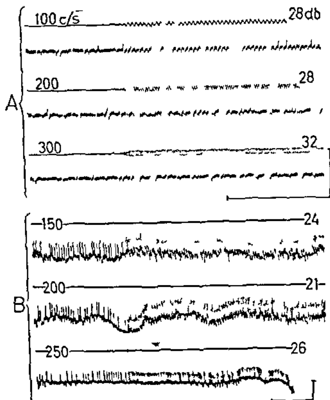


Fig 13 Two saccular units with spontaneous burst activity and different thresholds The non adaptive nature of the responses is clearly seen except for a slow and weak adaptation to 250 cps in *B* In *A* 300 cps at 32 db barely elicits a response *B* is a d c record ng and fluctuations of the baseline were caused by movements of the fish Vertical bar 2 mV time bar 250 msec

activity the curves for frequency-dependent threshold alterations ran rather parallel as seen from Fig 15 In units without spontaneous activity (Fig 15 *A*) the threshold rose by 6–14 db per 100 cps increase in sound frequency and in units with irregular spontaneous activity (Fig 15 *B*) by 3–8 db per 100 cps (These values represent extremes and do not express a linear relationship between increase of threshold and increase of sound frequency) Units with spontaneous burst activity had threshold minima around 150 cps i e both lower and higher frequencies required higher intensities to elicit a response (Fig 15 *C*)

In Fig 16 is plotted the rate of threshold increase versus the rate of

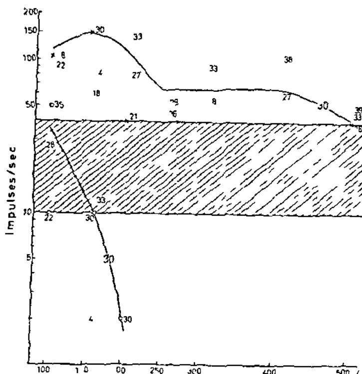


Fig 14 Relation between rate of adapted response and sound frequency for the two units in Fig 12 x = Unit *A* irregular spontaneous discharge averaging 20–30 spikes/sec Hatched area represents extremes obtained by counting impulses/100 msec o = Unit *B* no spontaneous activity Nos on points = sound pressure in db above 1 μB Curves = estimated response at 30 db intensity at each frequency

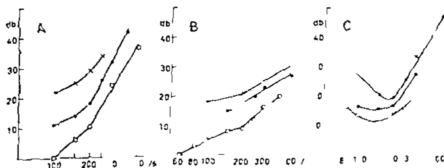


Fig 15 Relation between sound frequency and threshold *A* no spontaneous activity *B* irregular activity *C* burst activity Bottom curve of *A* was a unit with a low rate of spontaneous discharge

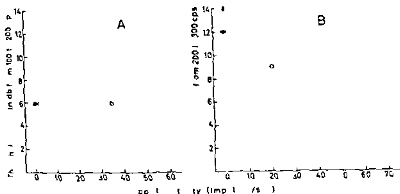


Fig 16 Relation between spontaneous dis harge rates and threshold increase per 100 cps increase in sound frequency ● = units without spontaneous activity x = units with irregular activity o = units with irregular activity and low sound thresholds

spontaneous discharge for two frequency steps namely 100 to 200 (*A*) and 200 to 300 (*B*) cps The rise of threshold per 100 cps was greatest for units without spontaneous activity and became gradually lower with increasing spontaneous discharge rates But between units with and units without spontaneous activity the transition was abrupt and a distinction between the two types seems consequently also valid from a functional point of view For units without spontaneous activity the average rise in theshold was 6 8 and 12 0 db for a frequency increase from 100 to 200 and from 200 to 300 cps respectively For units with irregular spontaneous activity the corresponding values were 3 8 and 6 1 The difference between the two types of units was statistically highly significant ($p < 0.005$) as tested by the method of WELCH (1951) assuming that the threshold rises for both types were independent and normally distributed

At moderate sound pressures units with spontaneous discharge responded to both low and high frequencies while units without spontaneous activity responded to low frequencies only as already shown in Fig 12 and 14 However the two cannot really be distinguished on the basis of spontaneous discharge rates alone since what is here called spontaneous activity was in many cases fully or partly response to low frequency background noise The slope of the threshold curves (Fig 15 4 *B*) or the threshold rise per 100 cps increase in frequency (Fig 16)

seems to be a better criterion for differentiating the two types of units In Fig 15 *A* the bottom curve represents a unit with a low rate of spontaneous discharge and the upper curves units without spontaneous activity One would not hesitate to assume that they were of the same type but that the bottom unit responded spontaneously to background vibrations due to its low threshold Likewise part of the spontaneous activity of the bottom unit in Fig 15 *B* was response to background noise

This view is also supported by Fig 16 in which the open circles denote values for increase of threshold per 100 cps in units with thresholds below 10 db These values correspond to those for units without spontaneous activity or with spontaneous discharge rates far below those actually recorded indicating that the spontaneous activity – fully or partly – was response to background vibration Complete absence of background noise would probably reveal a clear distinction between the two types of units namely those with *no* spontaneous activity and those with *true* spontaneous activity

The lowest threshold ever obtained for a 100-cps tone was about 4–6 db below reference level In Fig 15 *A* and *B* are plotted units which had thresholds just at reference level for 100 and 60 cps respectively

Inasmuch as the dorsal side of the fish s skull was above the water surface and the total sound pressure gradient encountered by the fish s head was 10–12 db any estimate of the absolute threshold sound pressure might seem to be of no value However the extra-cranial structures seem to be unimportant as sound conductors When the fish was elevated so that the neurocranium was out of water the sound response was abolished or drastically reduced The neurocranium is around 1.5 cm high and encountered a pressure gradient of roughly 6 db not *counting the top part of the skull which was above the water* The hydrophone presumably measured the sound pressure at 1 cm depth (it lay just under the surface and had a diameter of 2 cm) which was the same as the depth of the otoliths Whether this measured sound pressure was really the same as the average sound pressure through the skull is hard to tell but the difference between the two was probably not more than 2–6 db the measured pressure being the greater On this basis the threshold for *Cottus* to sound frequencies of 60–100 cps is estimated *to be 7–12 db below 1 μB* i e 0.5–0.3 μB which is about 6 db above that found for the eel (GRIFFIN 1950)

The *sensitivity* defined here as the increase in discharge rate per db increase in sound pressure is a parameter applicable only to units without spontaneous activity and *units with irregular activity* because burst activity units displayed the following response Its range was 2–14

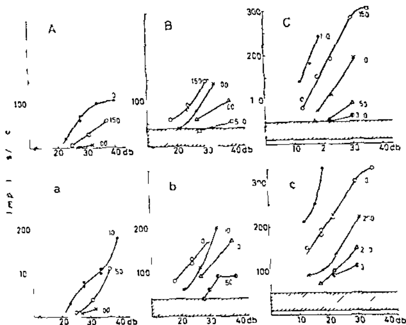


Fig 17 Relation between discharge rates and sound intensity at different frequencies (indicated on each curve) *A* *a* unit without spontaneous activity *B* *b* and *C* *c* units with irregular spontaneous activity *A–C* adapted responses *a–c* the corresponding initial responses during the first 100 msec of stimulation Note that unit *C* *c* has low threshold.

impulses/db/sec for the adapted response the lower sound frequencies having the higher values (Fig 17) The sensitivity decreased with increasing frequency but less rapidly in units with than in units without spontaneous activity as seen in *A* and *B* In *C* is plotted a unit whose spontaneous activity must have been due at least partly to background noise because its threshold was low Its sensitivity to low frequencies was high but with increasing frequencies rapidly dropped to values below those of irregular activity units with higher thresholds seen by comparing *B* and *C* This unit was exceptional in its high discharge rates A 150-cps tone elicited a prolonged double following response which makes it the more remarkable that the response to 300 cps was rather weak On the other hand this shows 1) that the sensitivity was independent of the spike frequency as such as is also illustrated in Fig 17 *B* and *C* where the slopes of curves for 150 cps are equal but dis-

charge rates different and 2) that the rate of decrease in sensitivity with increasing frequency was not dependent upon the spontaneous discharge rates actually recorded but on *true* spontaneous activity Thus the distinction between the two made on the basis of threshold differences is supported by the data on sensitivity

To show that the changes in sensitivity were not a matter of varying rates of adaptation the initial phases of the responses counted as number of impulses during the first 100 msec were plotted in Fig 17 *a* *b* and *c* It can be seen that except for the higher frequencies the slopes of the curves are approximately the same for the initial as for the adapted responses and the difference between units with and without spontaneous activity becomes even more pronounced in the initial phase of the response (Fig 17 compare *A* with *a* and *B* with *b*)

Discussion

CLASSIFICATION OF SENSORY UNITS The sensory units of the acoustic nerve have been classified into four groups 1) units showing irregular spontaneous activity 2) units showing spontaneous burst activity 3) units showing regular spontaneous activity, and 4) units showing no spontaneous activity This classification was made according to the general appearance of the spontaneous activity but has also been justified by the analysis of the response to pure tone stimulation in which the four types of units showed different patterns of adaptation and different sound thresholds and sensitivities in relation to sound frequency

With the exception of burst-activity units the same categories have been used by TASAKI (1954) for guinea pig auditory nerve fibres by GALAMBOS and DAVIS (1943) and KATSUKI et al (1958) for cat and by LOWENSTEIN and ROBERTS (1949 1951) for elasmobranch labyrinth nerve fibres The discharge rates were as high as 200 spikes/sec for the cat and 100/sec for the guinea pig while for the ray there were seldom more than 30/sec This corresponds nicely with the present results on *Cottus* where discharge rates were usually below 40/sec although they could go up to 80/sec and in burst-activity units to 150/sec

The validity of this spontaneous burst activity has been carefully considered as one could suspect it of being caused by injury TASAKI (1954) mentioned burst activity but classified it as injury discharge His units lasted only a fraction of a minute however and the burst-activity units

were unresponsive to sound stimulation In the present investigation all types of units could last as long as an hour with no sign of deterioration If burst activity had been an injury discharge one would expect it occasionally to have been initiated during the recording from other types of units by accidental small movements of the electrode or the fish but this was never observed Burst activity is virtually absent in records from the lagenar units as compared with saccular and utricular units although the recording conditions were the same in all three nerve branches Furthermore the response of burst-activity units to sound is strikingly different from that of other units both with respect to adaptation and to threshold variations as a function of sound frequency Finally units with burst activity respond without exception to sound stimulation which is not the case with any other type of units Thus it must be concluded that spontaneous burst activity represents physiological activity in neurons associated with particular receptors in the teleost labyrinth

The origin and possible functional significance of the spontaneous activity in primary sensory neurons is of considerable interest (GRANIT 1955) and one of the present observations has a possible bearing upon this problem The similarity in time course between the recovery of the response to sound and the recovery of the spontaneous activity after prolonged stimulation suggests that these activities were generated by the same elements presumably the sensory cells

Histologically the sensory cells are of the same type in sacculus and lagena and it is tempting to correlate the lack of burst activity in the lagenar nerve with the fibre-diameter spectrum KRISTOFFERSEN and ENGER (in preparation) show that most of the fibres in this nerve were 4 μ or less in diameter while the saccular has about equal amounts of 2 4- 6- and 8-μ fibres If one assumes that comparatively thick fibres mediate the spontaneous burst activity (frequency of individual bursts 300-400 spikes/sec) burst activity would be rare in the lagenar nerve A similar argument does not hold true for the utricular nerve because its fibre spectrum is like the one for the lagena However the total number of fibres in the utricular branch is around 2000 in contrast to 1000 in the saccular and lagenar nerves Furthermore the sensory cells in the utriculus are of two types one like the cells of the sacculus and lagena and the other like the cells of the cupulae of the semicircular canals Unfortunately the fibre diameter spectra of the two sensory areas are not known

KATSUKI YOSHINO and CHEN (1950) found a correlation between fibre diameter and rate of spontaneous activity in the lateral line nerve of the Japanese eel (*Anguilla japonica*), thin fibres had higher spontaneous discharge rates and lower threshold to stimulation than thick fibres This contradicts the argument given above for the burst-activity units in *Cottus* but is in agreement with the finding that units with irregular activity had on the average a lower sound threshold than units without spontaneous activity The spontaneous discharge rates in the Japanese eel are comparable to irregular discharge rates in *Cottus* but the present study tells nothing about the fibre diameters

SOUND RESPONSE AND FREQUENCY DISCRIMINATION The fact that response to sound stimulation has been obtained both from pars inferior i e lagena and sacculus and pars superior i e utriculus is in agreement with results from previous conditioning experiments as described in the introduction The fact that relatively few (1/3) utricular units responded to sound while practically all saccular and most (2/3) lagenar units did so indicate it reasonable to regard the acoustic nerve response as a sign of activity in pathways associated with sound perception The present investigation thus provides additional support for the notion of the primary importance of the pars inferior in this function and at the same time supports the view that all labyrinthine end-organs are potential vibratory receptors and manifest themselves as such in fishes (DE BURLET 1935 LOWENSTEIN and ROBERTS 1951)

Although the present study does not elucidate the function of non-acoustic units it seems reasonable to regard these as having at least partly a vestibular function In elasmobranchs LOWENSTEIN and ROBERTS (1949) showed that units with a regular spontaneous discharge responded to positional changes and in *Cottus* it has here been shown that the same type of unit responded if at all only to low-frequency high-intensity sounds

The adaptation found in all but burst-activity units and the post-stimulatory depression of spontaneous activity found in all spontaneous-activity units have been seen also in mammalian and elasmobranchian auditory nerve fibres In mammals GALAMBOS and DAVIS (1943) found the depression or silent period to be followed by an after-discharge upon high intensity stimulation a phenomenon not observed by LOWENSTEIN and ROBERTS (1951) in elasmobranchs or by the present author However this discrepancy may very well be due to the fact that

Galambos and Davis (1943 1948) recorded from second order neurons

The lack of adaptation observed in all burst activity units can possibly be a function of their generally high spontaneous discharge rates although some units with irregular spontaneous activity were also non adaptive for low-frequency high-intensity sounds Lowenstein and Roberts (1951) point out the probability that adaptation is not shown by all sense endings to an equal degree Differences in adaptation rates could possibly rest on differences in the mechanical properties of the sensory cells Each neuron of the acoustic nerve innervates several receptor cells (Ramon Cajal 1908) and it is probable therefore that the response recorded from a sensory neuron is the composite response of a group of receptor cells

Whether or not there are any such differences in mechanical properties within such a group of cells is not known However in the tympanic organ of a noctuid moth there are two types of cells one of which adapts rather quickly and the other slowly during stimulation (Suga 1961) Autrum (1960) found a phasic and a tonic component of the response to sound stimulation in the tympanic nerve of a grasshopper It could be possible that a similar mechanism operates in fishes (and other vertebrates) in such a way that the initial phase of the response is a phasic component initiated by one type of sensory cell and the adapted phase originates in another type of cell The fact that the recovery times for the initial and the adapted responses were different in *Cottus* may support this view Depending on which of the two types are in the majority in a given group of receptor cells one would record different adaptation rates (or no adaptation) in the corresponding sensory neuron

Previous work has indicated the probability that pitch discrimination in fishes is based upon a synchronization between sound frequency and frequency of impulse discharge Lowenstein and Roberts (1951) found following response up to 120 cps and no response at higher frequencies in the elasmobranch labyrinth Dudok van Heel (1956) showed that pitch discrimination in minnows improved with an increase in temperature which he thought was due to a shorter refractory period of nerve fibres at higher temperatures and a consequent increase in ability to follow higher sound frequencies Schwartzkopff (1962) suggests that all vertebrates below mammals discriminate low and medium tone frequencies by synchronization of nerve impulses with sound frequency a theory originally proposed for mammals by Wever (1933 1949) This volley theory seeks to explain pitch discrimination

as an interplay between several neurons each one firing with a definite phase relation to the sound waves The discharge rate would depend upon both frequency *and intensity but a number of neurons should* together impose on the central nervous system a complete following response

From the present work there is no doubt that such a synchronization takes place in all types of units either during the initial or the adapted phase of the response *(adapting units) or throughout the stimulation* period (non-adapting units) Two or three spikes per sine-wave occurring at low frequencies and high intensities or the dropping out of impulses from a following response probably does not mask the synchrony pattern The non-adaptive burst-activity units illustrate the *volley theory nicely as they show complete following responses up* to 200–300 cps at moderate sound pressures as do also those irregular-activity units whose discharge rates never exceeded the frequency of the sound but it remains to be demonstrated that the frequency-analyzing structures of the central nervous system of fish employ the following response as such

On the other hand frequency discrimination based on the volley mechanism does not seem to be very elaborate in fish In Ostariophysi species pitch distinction is absent above 1000–1200 cps (DIJKGRAAF and VERHEIJEN 1950) In nonostariophysids it is reduced from a 9 per cent to a 12–15 per cent frequency difference when pitch increases from 150 to 300 cps and to more than a 33 per cent frequency difference above 600 cps (DIJKGRAAF 1952) This reduction cannot be explained by the volley theory unless one assumes *that the central nervous system is* so poorly developed that it can handle only a complete following response in one or each of several peripheral neurons

The results of the present investigation also suggest that additional mechanisms have to be taken into consideration in attempts to account *for the pitch discrimination of teleosts The response to sound stimu*lation seems more complicated than one would expect from the morphology of the fish ear and more complicated than necessary for a pitch discrimination based on the volley principle *It* has been shown here that two types of units those without spontaneous activity and those with irregular activity cover different frequency ranges Both responded to low frequencies but only units with spontaneous activity responded to high frequencies in such a way that those units with the highest spontaneous *discharge rates* most likely respond

to the highest frequencies perceived by the sculpin (which by comparison with other comparable nonostariophysids are probably 500–800 cps) It has also been shown moreover that the rate of rise in threshold per 100-cps increase in sound frequency is a better criterion for differentiation of the two types than the presence or absence of spontaneous activity alone (The response to background noise may namely mask the *true* spontaneous activity or absence thereof) Of two given units – one of each type – with the same threshold for a 100-cps tone – neither will respond to a 300-cps tone of the same intensity and only the irregular activity unit will respond if sound pressure is increased by 10–20 db More than 20 db increase in intensity is required to elicit a response in the unit without spontaneous discharge

It seems obvious then that these two types of units together could provide information on the frequency of sound The frequency area of each unit is broad as is also the case in mammals (TASAKI 1954 KATSUKI et al 1958) The sharpening of the response areas is performed centrally in mammals and it is likely that a similar although far less developed mechanism is a play in fishes It is difficult to understand why units with irregular spontaneous activity *per se* should respond to higher tones than those without but KATSUKI et al (1958) also reported that in the cat units with high spontaneous discharge rates responded to high tones whereas units with low-frequency spontaneous activity responded to low tones In mammals with a well developed cochlea as a peripheral frequency analyzer the necessity for such a mechanism seems obscure and leads one to speculate whether it has simply persisted after the evolution of more effective ones

The great difference in the hearing range between the Ostariophysi (and other fish with special sound conducting structures) and the non-ostariophysids is due to the presence of elaborate resonators and sound conductors in the former VON FRISCH (1938) states that the swim-bladder labyrinth connection increases the acuity of hearing SCHNEIDER (1942) reports that the upper limit of hearing is reduced from 2607–4645 to 652 cps on elimination of the resonator of anabantid fishes The extirpation of the malleus of the Weberian ossicles reduces the sensitivity by 30–40 db (POGGENDORF 1952) in the catfish and mutilation of the swimbladder reduces the sensitivity from 13 db (at 330–750 cps) to 30 db (at 1500 cps) (KLEERFKOPER and ROGGENKAMP 1959)

However the superior ability of the Ostariophysi to discriminate pitch cannot be explained by their extra labyrinthine hearing structures

nor can one suggest that they *have a morphological* discriminator Their labyrinth deviates from that of other fish mainly in the relative size of sacculus and lagena which is apparently of no importance for tone discrimination VON FRISCH (1938) found that both these receptor areas responded to the entire sound frequency range in two Ostariophysids and in the present study irregular-activity *units* (*which responded to* all frequencies assumed to be perceived by *Cottus*) were found in the saccular lagenar and utricular nerve branches (There remains a possibility that pitch discrimination could take place between sacculus-lagena *and utriculus as the latter responds only to low frequencies* (VON FRISCH 1938 DIJKGRAAF 1952) but it is unlikely that a structure whose main function is equilibrial should play an important role in tone distinction)

On anatomical grounds then it seems very probable that the peripheral neural mechanisms for pitch discrimination are similar in both Ostariophysi and nonostariophysids but that *units* with irregular spontaneous activity cover a much broader frequency range in the former One would expect such units with high discharge rates to respond to all frequencies perceived while units with low discharge rates would *cover low frequencies only For both types of fish, the peripheral tone* distinction will be crude and finer discrimination must then take place centrally The presence of generally well-developed acoustic lobes in the Ostariophysi *supports such a hypothesis*

Summary

The purpose of this investigation has been to analyse the electrical activity of single sensory neurons in the acoustic nerve of the *sculpin (Cottus scorpius)* in order to elucidate the neural mechanism underlying hearing and pitch discrimination in teleosts

A specially designed fish-holder and a non-resonant experimental tank allowed extracellular single unit recordings with *microelectrodes from the* acoustic nerve of the fish Stimuli were usually delivered as tone pips of 1–2 sec duration with frequencies of 60 to 500 cps

Single neurons have been classified into four groups according to the general appearance of their spontaneous activity 1) *irregular-* 2) burst-and 3) regular-activity units and 4) units not showing spontaneous activity All four types were found in each of the three nerve branches (*utricular* saccular and lagenar) but burst-activity units were rare in the lagenar branch

Response to pure tone stimulation was obtained from almost all saccular units from two-thirds of the lagenar and one third of the utricular units Units with regular spontaneous activity generally did not respond to sound All units with burst activity and units without spontaneous discharge responded to sound while irregular activity units might be either responsive or non responsive The average sound pressure threshold was lower for responsive irregular-activity units (12 db above 1 μB) than for units with burst activity or no activity (21 db)

In all but burst activity units the response consisted of an increase in discharge rate which was highest during the initial phase of stimulation and adapted to a fairly steady level within 200 msec Burst activity units showed no adaptation

Tone pips separated by a 2 sec interval elicited the same response each time but after a 1 min stimulus spontaneous discharge rates and the subsequent response to 1 sec tone pips were depressed Recovery times for the spontaneous activity and the adapted phase of the response were similar while the initial phase of the response reached its normal level in a shorter time

The size of the response measured as impulses/sec was within certain limits proportional to the logarithm of the sound pressure in adaptive units which were of 2 types 1) those discharging up to 2–3 spikes per sine wave period and 2) those whose discharge rates never exceeded the frequency of the stimulating sound In non adaptive units the response consisted of a disruption of the spontaneous burst activity into a following pattern

The relation between sound frequency and response differed among the three types of sound-responsive units Units not showing spontaneous activity responded to sound frequencies up to 200 cps units with irregular spontaneous activity responded to 300–500 cps and units with spontaneous burst activity showed following response up to 200–300 cps)

It is concluded that pitch discrimination in teleosts is based partly upon a following response mechanism according to the volley theory and partly upon a peripheral physiological analyser consisting of two types of sensory neurons those responding to low frequencies only and those responding to the whole range of frequencies perceived by the fish

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CONTENTS

INTRODUCTION

In the eighteen thirties *Magendie* (1839) demonstrated to his audience that a probe inserted into the basal brain ganglia of a rabbit made it run forwards Towards the end of the century experiments with the newly developed method of electrical stimulation of discrete brain regions in anesthetized animals were interpreted to indicate that the caudate nucleus has no motor function However the corpus striatum was again generally assumed to have a motor function after the hepato-lenticular syndrome was described by *Wilson* (1912) other nuclei were believed to work together with the corpus striatum to form the striatal (*Vogt* and *Vogt* 1919) or extrapyramidal system (*Jacob* 1923) The results of research on the corpus striatum since then are difficult to integrate into a concept or theory I have brought together in this survey the evidence from animal experiments As a general conclusion I can however state vaguely that the corpus striatum seems to function on the highest level of nervous integration Those who in the future may elucidate the function of the corpus striatum will be a long way towards understanding the most subtle mechanisms of the brain

TERMINOLOGY

In the literature the following terms are used as synonyms of corpus striatum or its parts

Basal ganglia May or may not include the amygdala or thalamus or both

Striatum May refer to the caudate nucleus putamen and globus pallidus, but may also refer to only the caudate nucleus and putamen as opposed to the histologically different *pallidum*

Striopallidum is synonymous with corpus striatum

Neostriatum May refer to the caudate nucleus and putamen in mammals as opposed to the phylogenetically older *paleostriatum* i e globus pallidus Neostriatum and paleostriatum may also refer to homologous structures in reptiles and birds

Lenticular nucleus Putamen and globus pallidus

Striatal system May be a synonym of corpus striatum May also include a number of other, usually unspecified brain structures In the last usage it is interchangeable with the extrapyramidal system

Striate May refer to corpus striatum, the striatal system or area striata in cortex

The term corpus striatum in this survey denotes the caudate nucleus putamen and globus pallidus of mammals

Chapter I

THE 'EXTRAPYRAMIDAL SYSTEM'

An outline of clinicopathological investigations of the corpus striatum up to about 1930 may clarify the development of the concept of the ex trapyramidal system Reviews of clinicopathological studies were pre sented by *Jung* and *Hassler* (1960) and *Denny Brown* (1962)

A. DEDUCTIONS FROM CLINICOPATHOLOGICAL INVESTIGATIONS

Theories about the function of the corpus striatum were mentioned by *Charcot* (1877) but in his opinion reliable evidence was lacking *Ferrier* (1876) including the peduncular fibers in the corpus striatum stated that lesions of these structures in man "produce hemiplegia of the op posite side sensation remaining unimpaired According to *Gowers* (1887) however lesions of corpus striatum cause no lasting symptoms if limited to the grey matter

The first case of what became known later as the syndrome of the corpus striatum was published by *Anton* (1896) a boy with involuntary movements of all parts of the body had at autopsy, bilateral lesions con fined to the putamen In the years to follow many attempts to define a syndrome of the corpus striatum failed because the reported cases were not clear cut clinically or anatomically (for references see *Wilson* (1912)) The syndrome of the corpus striatum in the still current sense (*Grinker* and *Bucy* 1951 p 296) was described by *C Vogt* (1911) as athetose double and by *Wilson* (1912) as progressive lenticular degeneration In both conditions there were involuntary movements spasmodic contrac tures dysarthria and emotionalism without true paralysis or sensory change In Wilson s disease the involuntary movement was tremor rather than athetosis and involuntary associated movements were absent *Wilson* (1912) noted that paralysis agitans resembled the syndrome of the corpus striatum Although Huntington s chorea is characterized by both wide spread cortical lesions and lesions in corpus striatum (*Alzheimer* 1911) the dyskinesia of the disease was attributed to the lesion in corpus stria tum (*Vogt* and *Vogt* 1920)

The aim of later investigations was to establish in detail the relationship

between different sites and types of lesions in the corpus striatum and clinical manifestations However, the lesions were not confined to the corpus striatum in cases similar to those described by *Vogt* and *Wilson* in other respects The damaged structures were believed to constitute a system, and lesions in corpus striatum or the striatal' system were believed to be the cause of involuntary movements Assuming that abnormal movements are caused by lesions in the striatal system' there are two ways the movements may arise abnormal impulses may be discharged by the damaged neurones producing chorea athetosis etc (*Kahler* and *Pick* 1879), or the neurones may have been inhibitory before they were damaged, the resultant release accounts for the abnormal movements (*Jackson* 1884) *Anton* (1896), *Pineles* (1899), *Bonhoeffer* (1901), *von Economo* (1910), *Wilson* (1912) *Kleist* (1918) and *Vogt* and *Vogt* (1920) believed that chorea and athetosis were too well coordinated to be the result of random nervous impulses Furthermore the symptoms often remain unchanged during decades while the necrobiotic process progresses Therefore it was generally believed that the normal function of corpus striatum was to inhibit movement

Other theories of the pathophysiology of extrapyramidal motor disease were proposed *Bonhoeffer* (1901) and *von Economo* (1910) suggested that chorea is due to imbalance of pyramidal and other motor impulses *Wilson* (1912) concluded that corpus striatum has a steadying effect on the action of the corticospinal system and suggested that tremor is normally prevented via the rubrospinal tract and that rigidity is prevented via thalamus and cortex *Kleist* (1918) revived the idea of *Pineles* (1899) of a possible role of the cerebellum in the mechanism of chorea, and proposed an inhibitory circuit from cerebellum through the red nucleus to thalamus and corpus striatum, an interruption of this circuit producing chorea From a study of 34 cases of striatal disease *Vogt* and *Vogt* (1919, 1920) concluded that globus pallidus, in agreement with its simple structure, was a simple motor center emitting excitatory impulses whereas the caudate nucleus and putamen were believed to be coordinating centers emitting inhibitory as well as excitatory impulses (compare histology p 32) Lack of inhibitory impulses from the caudate nucleus and putamen to globus pallidus were thought to produce tremor when the damage was slight chorea when the damage was severe Akinesia on the other hand was explained by lack of excitatory impulses from the caudate nucleus and putamen to globus pallidus or by destruction of the globus pallidus itself *Foerster* (1921) too maintained that specific clinical pictures corresponded to lesions in different parts of the striatal system although the etiology could differ in cases with the same signs

With *Jacob* (1923 1925) the theory of extrapyramidal disease be came rigid and elaborate The syndrome of the corpus striatum was split up into striatumsyndrome and a pallidumsyndrome Damage to the caudate nucleus and putamen was claimed to produce chorea if mainly the small cells were destroyed rigidity if the large cells degenerated as well Partial destruction of globus pallidus was held responsible for athe tosis complete destruction for severe rigidity with contractures Parkin son s disease was believed to be a syndrome of the nucleus niger As re gards normal physiology *Jacob* assumed that the caudate nucleus and putamen were centers of emotional expression fear and flight orientation and attention reactions automatic adjustments of posture and position automatic associated movements sitting standing walking mastication swallowing and speech The globus pallidus was thought to be the center of synergies of smaller groups of muscles Jacobs presentation was based on the work of *Vogt* and *Vogt* (1919 1920) but unlike the *Vogts* *Jacob* mixed facts and speculations

Lewy (1921) criticised current theories of the pathophysiology of the dyskinesias and stated that the ideas of most authors were viel zu grob und einfach The evidence behind the theories was *insufficient* because patients with well-defined syndromes have different lesions at autopsy and lesions confined to the corpus striatum may lead to different clinical signs (for further references see *Spatz* 1927 *Wilson* 1929 *Rioch* 1940 *Meyers* 1953 *Greenfield* 1957)

B DEVELOPMENT OF THE CONCEPT OF THE "EXTRAPYRAMIDAL SYSTEM

The concept of the extrapyramidal system has developed gradually no single author invented it[1]) *Wilson* (1912) introduced the term extrapyra midal motor disease with the following remark It is unfortunate that there is no expression in common use to indicate extrapyramidal motor disease He referred to diseases of corpus striatum and its efferent path ways via the red nucleus thalamus and cortex In this and later stu dies the hypothesis was implied that the involuntary motor activity of normal individuals can be subdivided into components each gover ned by a special extrapyramidal nucleus *Wilson* (1929) realized this view was too simple "Voluntary movements were assigned to the pyramidal system because lesions in the internal capsule produced hemiplegia *Kleist* (1918) described a *subcortical motor system* which in

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cluded the dentate nucleus *Vogt* and *Vogt* (1920) defined a *striatal system* as a system of brain structures so intimately interrelated with corpus striatum that lesions in any part of the system evoke the syndrome of the corpus striatum In its widest sense the striatal system of the *Vogts* consisted of the corpus striatum the nuclei ruber, subthalamicus niger, interstitialis and Darkschewitschi, and part of the thalamus *Jacob* (1923) first used the term extrapyramidal system defined by the appearance of extrapyramidal disturbances of movement when the system was damaged The argument was circular in that extrapyramidal disturbances of movement were defined as symptoms that appeared in diseases of certain parts of the basal ganglia Despite the critical attitudes of *Spatz* (1927) and *Wilson* (1929) the extrapyramidal system continued to be a hazy concept based on speculation rather than evidence The subdivision of the motor functions of the brain into pyramidal and extrapyramidal functions has lost its foundation because damage to the pyramidal tract does not elicit the pyramidal syndrome of clinical neurology Homolateral spasticity is produced by hemidecerebration in cats (*Sherrington* 1898) and after section of the medullary pyramid in cat dog, monkey and chimpanzee (*Schiff* 1858, *Starlinger* 1896 *Rothmann* 1907, *Marshall* 1933, 1936, *Tower* 1944) or the cerebral peduncle in monkey (*Cannon, Magoun* and *Windle* 1944 *Bucy* and *Keplinger* 1961) and man (*Walker* 1952 *Meyers* 1956 *Bucy* 1957, *Bucy* and *Keplinger* 1961) spastic hemiplegia is absent

An anatomical definition of the extrapyramidal system depends on the definition chosen for the pyramidal system Literally the pyramidal *tract* is the lengthwise running fibers on the ventral side of the medulla oblongata But cortico-bulbar fibers outside the pyramid may play a role similar to that of the cortico-spinal tract and fibers from the pyramids terminate in the reticular formation (for references see *Patton* and *Amassian* 1960) Nevertheless the term pyramidal tract could mean the corticospinal tract without leading to confusion (*Tower* 1944) The cortical region of origin of the tract is included in the concept of the pyramidal *system* Nearly all the fibers in the pyramids degenerate after ablation of the anterior third of the cerebral cortex in cats (*van Crevel* 1958) and areas 4 6 and parietal cortex in monkeys (*Russell* and *de Myer* 1961) But the motor cortex has also extrapyramidal projections (*Rothmann* 1907) Thus pyramidal and extrapyramidal systems overlap A further difficulty is that the extrapyramidal system defined as central motor mechanisms excluding the pyramidal system or tract has vague boundaries (*Jung* and *Hassler* 1960) Motor mechanisms cannot be delineated anatomically and motor effects are obtained from the whole brain if it is not anesthetized

To assign a specific meaning to the term extrapyramidal system *Rioch* (1940) suggested that it be used for fiber systems that originate in the forebrain and terminate within the brain In contrast pyramidal fiber systems terminate at the level of the anterior horn cell Extrapyramidal systems are thus interrelated high upstream from the final common path (*Rioch* 1940) The functional significance of this distinction is unknown Therefore and as the terms pyramidal and extrapyramidal have strong older connotations, a redefinition does not seem appropriate

Meyers (1953) recommended that the term extrapyramidal system be discarded claiming that the concept from its beginning was a violation of logic and common sense Others use the term extrapyramidal system without a specific definition with reference only to convention (*Jung* and *Hassler* 1960)

In short the concept of the extrapyramidal system in its original sense is no longer useful because the hypothesis it referred to has not been confirmed To redefine the extrapyramidal system may lead to misunderstandings since older definitions although vague are in common use

On the other hand the dichotomy expressed by the terms pyramidal and extrapyramidal motor disease is real (*Wilson* 1912) Motor syndromes caused by diseases of the brain are usually either spastic hemiplegia with hyperactive tendon reflexes the sign of Babinski and absent abdominal reflexes or they consist of various combinations of chorea athetosis tremor and rigidity Referring to these syndromes as pyramidal and extrapyramidal has lost its original meaning Correct anatomical or pathophysiological names probably cannot be found because the damaged mechanisms are too complex Artificial names rarely catch on To abandon the concept of the extrapyramidal system while we continue to speak about the extrapyramidal syndrome will present difficulties but we may have no realistic choice

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both the caudate and lenticular nucleus of monkeys anesthetized with ether and chloroform were electrically inexcitable Slight preference for the ipsilateral hand was the only effect of unilateral electrolytic lesions

Despite these mainly negative results of animal experiments clinico-pathological observations were interpreted to indicate that the function of the corpus striatum was motor (see p 14) Therefore motor effects of ablation or stimulation of corpus striatum were repeatedly reinvestigated Until recently the negative results were confirmed whenever involvement of neighbouring structures was sufficiently investigated However motor effects were found whenever ablation or stimulation was *not confined to* corpus striatum *Edwards* and *Baggs* (1923) found that lesions 1 cm in diameter made with radium tubes inserted in the caudate nucl us and putamen of dogs produced tremor lasting for about 4 days and disturbances of muscle tone lasting about 4 weeks The figures show that the lesions extended into the internal capsule Lesions in corpus striatum in sensorimotor decorticate oppossums added tonic flexion of head and fingers to the motor disturbances (*Rogers* 1924) Lesions of corpus striatum cortex internal capsule and mesencephalon in cats produced circling movements tremor and disturbances of tone lasting for about one week These effects were ascribed to the corpus striatum (*Morgan* 1927) The absence of motor effects of stimulation of corpus striatum when the internal capsule had degenerated was confirmed by *Spiegel* and *Takano* (1929) and *Ashwawa* and *Lewy* (1929) *Miller* (1936) reported bilateral flexion and rotation of the limbs elicited by electrical stimulation or strychninization of the caudate nucleus of cats anesthetized with barbiturates Histological verification of the site of the stimulated points was not reported The absence of motor effects of stimulation confined to corpus striatum was again confirmed by *Bard* and *Rioch* (1938) destruction by freezing of corpus striatum in decorticate cats produced no new functional deficits The effect of electrolytic lesions in putamen and globus pallidus of monkeys was reinvestigated by *Brown* (1939) who found tremor and reluctance to initiate voluntary movements An increase in tone was not observed unless cortical areas 6 and 8 were damaged as well Histological controls of the lesions were not reported

The next period in the history of corpus striatum began with new results of animal exp riments 1) Strychnine neuronography in monkeys was interpreted to indicate a suppressor circuit area 4s→caudate nucleus→globus pallidus→thalamus→area 4 (*Dusser de Barenne* and *McCulloch* 1938) Malfunction of this and similar circuits was believed to explain athetosis and tremor (Fig 1) and *Bucy* (1942) stated that "the facts are now falling together like well fitting pieces of an intricate jig saw puzzle However after about ten years th

Chapter 2

MOTOR FUNCTION

Both clinicopathological observations and animal experiments have been interpreted to indicate a motor function of the corpus striatum This survey deals with animal experiments *Investigations from before about 1940* are reviewed only briefly

A HISTORICAL OUTLINE

Before about 1880 a distinction was usually not made between the in ternal capsule and what is now designated corpus striatum *Magendie* (1839) found that destruction of corpus striatum' bilaterally in rabbits produced forward running movements Running backwards occurred after ablation of the cerebellum *Magendie*s observation was repeatedly con firmed and *Nothnagel* (1873) termed the running center in the caudate nucleus nodus cursorius' *Ferrier* (1876) elicited general muscular contraction on the opposite side of the body by electrical stimulation of the corpus striatum in a number of animals anesthetized with chloroform The body became flexed so that the head approximated the tail and the corpus striatum was believed to integrate the centers which were dif ferentiated in the cortex The stimuli used by Ferrier may have spread to the internal capsule since *Gliki* (1976) found that the *caudate nucleus* of rabbits anesthetized with morphine and ether was electrically inexcit able and that movements were elicited by stimulation of the internal capsule This observation was independently made by *Franck* (1878) and confirmed by *Minor* (1882) *Ziehen* (1890) *Wieting* (1891 *Schuller* (1901) and *von Bechterew* (1909) *Putamen* was considered functionally analogous to the caudate nucleus *Von Bechterew* assigned a motor function to the *globus pallidus* because stimulation of this nucleus produced con vulsive attacks even when the internal capsule had degenerated Blood pressure and respiration could be altered by stimulation of the globus pallidus and putamen other autonomic effects ascribed to globus pallidus by previous authors were found to be due to activation of or damage to neighbouring structures (*von Bechterew* 1909) *Wilson* (1914) found that

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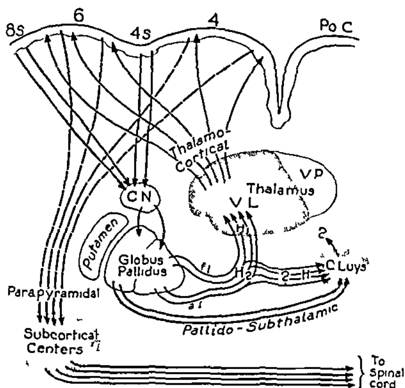


Fig 1

The mechanism of choreo-athetosis suggested by Bucy (1942) Areas 4s and 8s were believed to inhibit areas 6 and 4 through a circuit similar to the suppressor circuit described by *Dusser de Barenne and McCulloch* (1938) area 4s → nucleus caudatus → globus pallidus → nucleus ventralis lateralis of the thalamus → area 4 Interruption of the circuit should release areas 6 and 4 to abnormal activity resulting in the involuntary movements of choreo-athetosis The impulses responsible for the abnormal movements were thought to reach the spinal cord through the parapyramidal system defined as efferent fibers other than the pyramidal tract which arise from the precentral region

McCulloch (1938) was reinterpreted because electrical isolation of area 4s from area 4 prevented suppression, *Sloan* and *Jasper* (1950) showed that suppression had all the characteristics of spreading depression (*Leão* 1944) 2) Inhibition of movements in monkeys and cats was elicited by stimulation of the corpus striatum (*Mettler et al 1939*) Unlike older results of stimulation and ablation of the corpus striatum this finding was consistent with clinicopathological observations But inhibition of movements elicited from the corpus striatum could be elicited with a lower threshold from the internal capsule than from corpus striatum in nembutalized cats inhibition could not be elicited after degeneration of the descending fibers in the internal capsule (*Peacock* 1954)

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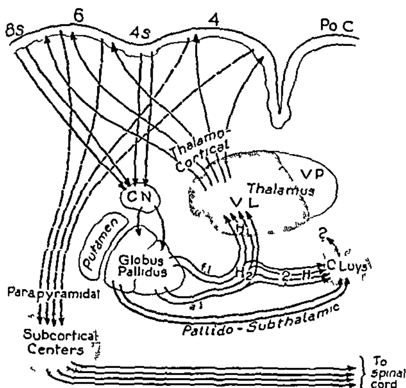


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Bilateral electrolytic lesions in the putamen of two monkeys produced a soft, yielding rigidity, poor placing and grasping reflexes and hyperactive righting reflexes In nine monkeys bilateral electrolytic lesions in globus pallidus, ansa lenticularis and the frontopontine tract produced a plastic rather than springy resistance to passive movements and absence of placing and righting reflexes These 'examples of reasonably restricted lesions did not produce tremor (*Denny-Brown* 1962)

Combined lesions of cortex and corpus striatum have been reported to produce motor effects, but there is no extrapyramidal syndrome as seen in patients In monkeys cerebral cortical lesions combined with lesions in the ipsilateral corpus striatum increased spasticity and abolished feeding movements in contralateral extremities (*Mettler* 1943) Bilateral lesions of the caudate nucleus added to bilateral ablations of cortex rostral to area 4 increased ambulatory hyperkinesia, and produced an increased tendency to climb The previously hyperactive, curious animal was transformed into an overactive 'automaton devoid of curiosity' (*Mettler* 1945 a) When the globus pallidus was included in the lesion associated move ments disappeared, particularly those called forth by proprioceptive stimulation' In monkey J 71 M both globi pallidi were included in the lesions, hyperkinesia was absent and the monkey did not alter a strange position it was passively placed in (*Mettler* 1943, 1945 a) *Kennard* (1944) too, found that spasticity produced by a cortical lesion was greater and more enduring when corpus striatum was damaged as well In addition there was bilateral tremor after uni and bilateral lesions in corpus striatum when these were combined with different cortical lesions regardless of whether area 6 8 or insular cortex were damaged Tremor was more pronounced and enduring in chimpanzee than in monkey *Kennard* (1944) did not report histological findings and it is therefore not known whether there was damage to mesencephalic areas which might account for the tremor (*Ward McCulloch* and *Magoun* 1948)

Rioch and *Brenner* (1938) investigated chronic decorticate cats guinea pigs and opossums and found no new functional deficit when lesions were made in the corpus striatum A similar study by *Mettler* and *Mettler* (1942) described several abnormalities in cats with combined lesions of cortex and corpus striatum but the animals died within 5 days after the operation In a note *Heath Freedman* and *Mettler* (1947) indicated that removal of up to 75 per cent of corpus striatum through different cortical lesions produced constant abnormalities, the cats were dull and assumed bizarre postural patterns' but muscle tone was normal This was in terpreted to indicate loss of contact with the environment and disturbances in proprioception

C STIMULATION STUDIES SINCE ABOUT 1940

1 Movements

Stimulation of the exposed ventricular surface of the caudate nucleus in a human anesthetized with nitrous oxide and pethidine evoked no movements (*Bates* 1954) This is in agreement with the results of experiments on anesthetized animals (p 18)

Stimulation of the caudate nucleus in alert animals has been reported to evoke movements of two kinds 1) contraversive head turning and circling (*Delmas Marsalet* 1925 *Hassler* 1956 *Buchwald* and *Ervin* 1957 *White* and *Himwich* 1957 *Stevens* *Kim* and *McLean* 1961 *Ruckebusch* and *Barone* 1961) and 2) flexion of contralateral extremities combined with contraversive head turning (*Forman* and *Ward* 1957)

It is not excluded that current spread to the internal capsule caused the flexion of the contralateral extremities elicited by stimulating electrodes in the caudate *Forman* and *Ward* concluded that contralat ral movements could still be elicited after ablation of the motor cortex on the side of stimulation but they did state (p 237) that flexion responses of the fore and hind limbs of the sort seen in animals with an intact cerebrum were absent in sensorimotor decorticate cats

It further remained uncertain whether contraversive h ad turning and cirling were elicited by activation of the caudate nucleus or a neighbouring structure (thalamus septum cortical efferent from areas outside the sensorimotor cortex)

When electrical stimuli are delivered to the corpus striatum pread of current to adjoining structures is unavo dable The caudate nucleus is situa ed between the internal capsule the nucleus ventralis anterior and nucleus reticularis of the thalamus the corpus callosum and area septalis Activation of these structures produces responses in other parts of the brain In cat and rhesus monkey the tip of an electrode in the caudate nucleus is at most 2 mm distant from one of its borders The putamen and globus pallidus are so small that an electrode in erted in these structures is within about 1 mm of the internal capsule or the amygdala Thus unless special precautions are taken it is difficult to ascertain whether responses in other parts of the brain are due to activation of the corpus s riatum or of adjacent structures

I have reinvestigated the movements elicited by stimulation of the caudate nucleus in alert cats before and after hemidecortication and before and after lesions in the caudate nucleus (*Laursen* 1962 a) The effect of spread of current to the internal capsule was futhermore determined with a 14-lead electrode thresholds were determined at points known distances apart without intervening displacements of brain tissue Contralateral flexion movements were elicited by stimulation between two adjacent electrodes of the multielectrode inserted through the caudate

Bilateral electrolytic lesions in the putamen of two monkeys produced a soft, yielding rigidity, poor placing and grasping reflexes and hyperactive righting reflexes In nine monkeys bilateral electrolytic lesions in globus pallidus, ansa lenticularis and the frontopontine tract produced a plastic rather than springy resistance to passive movements and absence of placing and righting reflexes These examples of reasonably restricted lesions' did not produce tremor (*Denny Brown* 1962)

Combined lesions of cortex and corpus striatum have been reported to produce motor effects, but there is no extrapyramidal syndrome as seen in patients In monkeys cerebral cortical lesions combined with lesions in the ipsilateral corpus striatum increased spasticity and abolished feeding movements in contralateral extremities (*Mettler* 1943) Bilateral lesions of the caudate nucleus added to bilateral ablations of cortex rostral to area 4 increased ambulatory hyperkinesia, and produced an increased tendency to climb The previously hyperactive, curious animal was transformed into an overactive automaton devoid of curiosity (*Mettler* 1945 a) When the globus pallidus was included in the lesion associated move ments disappeared, particularly those 'called forth by proprioceptive stimulation' In monkey J 71 M both globi pallidi were included in the lesions hyperkinesia was absent and the monkey did not alter a strange position it was passively placed in (*Mettler* 1943, 1945 a) *Kennard* (1944) too, found that spasticity produced by a cortical lesion was greater and more enduring when corpus striatum was damaged as well In addition there was bilateral tremor after uni and bilateral lesions in corpus striatum when these were combined with different cortical lesions re gardless of whether area 6 8 or insular cortex were damaged Tremor was more pronounced and enduring in chimpanzee than in monkey *Kennard* (1944) did not report histological findings and it is therefore not known whether there was damage to mesencephalic areas which might account for the tremor (*Ward McCulloch* and *Magoun* 1948)

Rioch and *Brenner* (1938) investigated chronic decorticate cats guinea pigs and opossums and found no new functional deficit when lesions were made in the corpus striatum A similar study by *Mettler* and *Mettler* (1942) described several abnormalities in cats with combined lesions of cortex and corpus striatum but the animals died within 5 days after the operation In a note *Heath Freedman* and *Mettler* (1947) indicated that removal of up to 75 per cent of corpus striatum through different cortical lesions produced constant abnormalities the cats were dull and assumed bizarre postural patterns but muscle tone was normal This was in terpreted to indicate loss of contact with the environment and disturbances in proprioception

C STIMULATION STUDIES SINCE ABOUT 1940

1 Movements

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It further remained uncertain whether contraversive head turning and cirling were elicited by activation of the caudate nucleus or a neighbouring structure (thalamus septum cortical efferent from areas outside the sensorimotor cortex)

When electrical stimuli are delivered to the corpus striatum spread of current to adjoining structures is unavoidable The caudate nucleus is situated between the internal capsule the nucleus ventralis anterior and nucleus reticularis of the thalamus the corpus callosum and area septalis Activation of these structures produces responses in other parts of the brain In cat and rhesus monkey the tip of an electrode in the caudate nucleus is at most 2 mm distant from one of its borders The putamen and globus pallidus are so small that an electrode inserted in these structures is within about 1 mm of the internal capsule or the amygdala Thus, unless special precautions are taken it is difficult to ascertain whether responses in other parts of the brain are due to activation of the corpus striatum or of adjacent structures

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nucleus and the internal capsule In the center of the head of the caudate nucleus the threshold was five times the threshold in the internal capsule Contraversive head turning and circling could not be elicited by stimulation between two adjacent electrodes of the multielectrode probably because too little of the tissue was stimulated (interelectrode distance 0 6 mm) This response together with contralateral flexion movements could be elicited by stimulation between the tips of two electrodes 2 mm apart After electrolytic lesions made with these electrodes contraversive head turning and circling were abolished, contralateral flexion movements could still be elicited After decortication flexion responses were abolished *whereas the head turning and circling response were unaffected*

These results indicate that contraversive head turning and circling are elicited by stimulation confined to the caudate nucleus and that contralateral flexion responses are due to spread of current to the internal capsule Stimulation at different frequencies also indicates that the head turning circling and the flexion response are elicited from different structures Flexion responses had the lowest threshold at 150/sec, contraversive circling at 50/sec Therefore either response could be obtained in the same animal without change in electrode position or in stimulus intensity (Fig 2) The above findings are in accordance with the effect of acetylcholine and diisopropylfluorophosphate applied to the caudate nucleus contraversive head turning and circling were elicited, not contralateral flexion responses (*White* and *Himwich* 1957 *Stevens Kim* and *McLean* 1961) In agreement with earlier investigations (p 18), the caudate nucleus was inexcitable one month after decortication in cats anesthetized with Nembutal® (*Hendley* and *Hodes* 1953)

Somatotopic organization of the corpus striatum was inferred from clinicopathological studies (for references see *Davidson* and *Goodhard* 1940) but was not confirmed in monkeys and apes (*Kennard 1944*) The somatotopic organization of the caudate nucleus observed by *Forman* and *Ward* (1957) in cats was probably the somatotopic organization of the internal capsule (*Laursen* 1962 a)

Recently *Montanelli* and *Hassler* (1962) found contraversive head turning and circling and movements of the contralateral legs elicited by stimulation of the globus pallidus and entopeduncular nucleus [*]) in alert cats Evaluation of this experiment must await publication of a full report

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*) The homologue in carnivores of the *medial segment of the* globus pallidus in primates

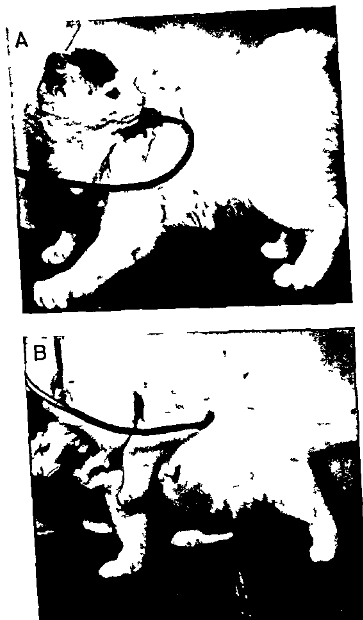


Fig 2

Differentiation of head turning and flexion responses by variation of the stimulation frequency (*Laursen* 1962 a) Implanted bipolar electrode in the right caudate nucleus Stimulation by pulse trains of 5 sec – A Contraversive (left) head turning without flexion of contralateral extremities stimulus strength 1 2 mA at 50 sec – B Flexion of contralateral (left) foreleg without head turning Stimulus strength 1.2 mA at 150 sec

2 *Inhibition of movements*

Two types of inhibitory reactions have been described as due to stimulation of the corpus striatum 1) immediate cessation of movement, 2) diminution or abolition of spontaneous movements after a latency of several minutes

1) In acute experiments electrical stimulation of the caudate nucleus and globus pallidus inhibited movements evoked from the cerebral cortex (*Mettler* et al 1939, *Freeman* and *Krasno* 1940, *Mettler* and *Mettler* 1941, *Gerebtzoff* 1941 *Hodes Peacock* and *Heath* 1951) However, *Peacock* (1954) showed that inhibition in nembutalized cats was elicited with weaker stimuli from the internal capsule than from the caudate In alert animals *Buchwald* and *Ervin* (1957) and *Buchwald* et al (1961 b) elicited immediate inhibition of motion by stimulation at 30–40/sec through electrodes in the caudate nucleus without, however, ascertaining that stimulation was confined to this nucleus *Forman* and *Ward* (1957) did not find inhibitory phenomena in alert cats

Because the evidence in the literature is conflicting I have investigated whether immediate inhibition of movements can be elicited in alert cats by stimulation confined to the caudate nucleus The cats were trained to perform an escape or avoidance jump over a hurdle separating the two compartments of a shuttle box (*Laursen* 1962 b) Inhibition was thus studied on a standard background of motor activity Using an implanted multielectrode for stimulation the threshold of arrest of hurdle crossing was lower in the internal capsule than in the caudate nucleus, the threshold increased with increasing separation from the internal capsule Hemidecortication on the side of stimulation abolished the inhibitory response Muscle action potentials evoked by stretch or by cortical stimulation in anesthetized and encephale isole cats were inhibited only when stimulating through electrode pairs situated in the internal capsule Thus there was no evidence of an inhibitory effect of caudate origin Immediate inhibition of movement can be elicited by stimulation of the thalamus (*Hunter* and *Jasper* 1949) or the amygdala (for references see *Gloor* 1960, p 1404) During this arrest response the animal remains standing on its feet as if it were frozen When stimulation is interrupted the animal resumes movement with no appreciable delay

In pigeons movements induced by strychninization of the optic lobes were inhibited by stimulation of the neostriatum the homologue of the caudate nucleus and putamen in mammals (*Machne* 1952)

2) There are many reports of inhibitory effects after stimulation of the caudate nucleus in cats at rates of 1–12/sec (*Hess* 1928 *Akert* and *Andersson* 1951, *Akert* 1952, *Stevens Kim* and *McLean* 1961, *Buch*

wald et al 1961 b) The evoked behavior was described variously as sleep an inactivation syndrome or a state of quietness and the latency was several minutes *Heath* and *Hodes* (1952) in a brief note described sleep in monkeys and in man induced by caudate stimulation An attempt to quantitate the inhibitory effect in cats was made by *Buchwald* et al (1961 b) caudate stimulation produced slowing and cessation of bar pressing *Knott Ingram* and *Correll* (1960) found no effect of caudate stimulation on bar pressing in cats

Experiments on delayed inhibitory effects are difficult to interpret be cause the response is non specific Sleep or sleep-like states may be elicited also by rhythmic low frequency stimulation of the basal forebrain areas (cat *Sterman* and *Clemente* 1962) the diencephalon (cat *Hess* 1928 1948) the brain stem reticular formation (cat *Favale Loeb Rossi* and *Sacco* 1961) the region of the solitary tract (cat *Magnes Moruzzi* and *Pompeiano* 1961) peripheral nerves (cat *Pompeiano* and *Sweet* 1962) and somatic receptors (dogs *Pavlov* 1928)

Delayed inhibitory effects have also been observed after chemical stimulation (1) To avoid stimulation of fibers crystalline choline esters were deposited in the caudate nucleus Quietness and drowsiness were induced after five or more minutes (*Stevens Kim* and *McLean* 1961) This experiment is difficult to interpret because small doses of acetyl choline have an excitatory larger doses a blocking effect (*Buchthal* and *Lindhard* 1937) (2) Four to seven days after alumina cream was injected into the caudate nucleus in cats (*Spiegel* and *Szekely* 1961) the animals lapsed into a catatonic state this could be prevented by extirpa tion of the amygdala not by removal of the sensorimotor cortex nor by lesions in the globus pallidus This was interpreted to indicate an inhibi tory pathway from the caudate nucleus to the periphery via the amygdala There is no anatomical evidence for such connections An alternative explanation might be that catatonia was produced by stimulation of the nucleus ventralis anterior of the thalamus and that this nucleus in con junction with the amygdala was responsible for the phenomenon This interpretation is supported by the long latency of the catatonic state and by the fact that arrest reactions resembling this state can be elicited by electrical stimulation of nucleus ventralis anterior of the thalamus (*Hunter* and *Jasper* 1949) the amygdala (*Kaada* 1951) and the internal capsule (*Laursen* 1962 b)

3 *Effects on spinal reflexes*

a) *Gamma efferents and root to root reflex response* Stimulation of the medial part of the caudate nucleus accelerated spontaneous discharge

from muscle spindles (*Granit* and *Kaada* 1953), whereas internal capsule stimulation was inhibitory The cats were anesthetized with 10 mg Dial® and 20 mg chloralose per kg (*Eldred Granit* and *Merton* 1953) Stimulation of the posterior part of the globus pallidus in lightly nembutalized cats (dose not reported) enhanced stretch reflexes and movements evoked from cortex (*Peacock* and *Hodes* 1951) In agreement with this repetitive or single shock stimulation of all parts of the corpus striatum (and claustrum) increased the amplitude of the contralateral root to root reflex response in cats immobilized with Flaxedil® Potentials produced in ventral roots by systemic application of strychnine were increased as well (*Segundo Migliaro* and *Roig* 1958) These authors investigated the possible role of spread of stimulating current to the internal capsule in two cats partially decorticated three weeks previously and found essentially the same as in intact animals In deteriorated preparations stimulation occasionally produced a decrease in response amplitude as did small doses of barbiturates (2 mg/kg barpental[3]), larger doses (10 mg/kg) abolished spinal effects of stimulation of corpus striatum These observations were confirmed by *Stern* and *Ward* (1962) who found the same effect of stimulation of globus pallidus and nucleus ventralis lateralis of the thalamus the possible effect of spread of current to the internal capsule was not investigated The spindle afferent discharge from extensors of the hind foot of nembutalized cats (30 mg/kg) was inhibited by stimulation of contralateral globus pallidus and nucleus ventralis lateralis of the contralateral thalamus (150/sec *Stern* and *Ward* 1960) In lightly nembutalized cats (15–25 mg/kg) spindle afferent discharges from both flexors and extensors were enhanced by stimulation of the contralateral globus pallidus internal capsule, and caudate nucleus In anesthesia of moderate depth (after an additional dose of 7 mg/kg) the discharge from flexors was still enhanced but in agreement with *Stern* and *Ward* (1960) the discharge from extensors was inhibited (*Shimazu Hongo* and *Kubota* 1962)

Contrary to the above results *Sasaki Namikawa* and *Matsunaga* (1960) reported a decrease of the root to root reflex response and hyperpolarization of both flexor and extensor motoneurons elicited by stimulation of the caudate nucleus and globus pallidus The cats were anesthetized 5 to 7 hours before the experiment with 25 mg/kg of Nembutal® The stimulus consisted of brief trains of 0 05 to 0 10 msec rectangular pulses at a frequency of 5000 to 10000/sec

In his book on the neurophysiology and neurochemistry of convulsion *Hayashi* (1959) mentions that extensor hypertonus, produced in dogs by damage to the grey substance around the aqueduct was abolished by

[3]) Sodium 5 ethyl 5 (1 methylbutyl) barbiturate

shocks from an induction coil or injection of nicotine in the caudate nucleus Other details of the method are not given

b) *Autonomic reflexes* The galvanic skin response to stimulation of the central stump of a cut nerve was reported to be reduced during monopolar stimulation of the caudate nucleus (*Wang* and *Brown* 1956) The cats were decorticated at the beginning of the experiment but spread of current to the internal capsule was not excluded because time was not allowed for degeneration of fibers The gastro-intestinal tract and the bladder in cats responded to stimulation of a number of structures at the base of the brain including the globus pallidus and putamen The role of spread of current was not evaluated (*Strom* and *Uvnas* 1950) Autonomic responses attributed to stimulation of the caudate nucleus by *Strom* and *Uvnas* (1950) *Akert* and *Andersson* (1951) *Wall Glees* and Fulton (1951) *Buchwald* and *Ervin* (1957) *Wang* (1960) and *S ekely Kirby* and *Spiegel* (1961) are probably due to activation of area septalis (*Laursen* 1962 a)

Although stimulation confined to the corpus striatum thus may be without immediately observable autonomic effects globus pallidus may have a function related to metabolism cats with bilateral lesions in the globus pallidus become sluggish (*Laursen* 1962 c 1963) They do not eat and hardly move the first four or five days after the operation About two weeks after the lesions are made the cat s behavior is again normal without the apprehensiveness which some show before the operation The slow recovery is not a non specific effect of the surgical intervention because cats with bilateral thalamic lesions behave normally two days after the operation Similarly rats do not eat or drink after lesions in the pallido-fugal tracts (*Morgane* 1961) and caudate nucleus (*Whittier* and *Orr* 1962) and monkeys recover slowly after amygdala l sions (amygdala hangover *Weiskrantz* 1956)

SUMMARY

Lesions confined to the corpus striatum have no effect on motor activity in animals including primates Lesions in the corpus striatum combined with lesions in the cortex or internal capsule produce spasticity akinesia and tremor Associated damage in the mesencephalic tegmentum may be responsible for the tremor

Stimulation of the head of the caudate nucleus produced head turning and circling to the side opposite the side of stimulation The cat looks as if it were searching something Stereotyped contralateral flexion responses are elicited by stimulation through electrodes in the caudate nucleus by

spread of current to the internal capsule The somatotopic organization assumed from the finding of foreleg responses or hindleg responses is in the internal capsule, not in the caudate nucleus

Immediate inhibition of movements (arrest reaction) elicited by stimulation through electrodes in the caudate nucleus is also due to spread of current to the internal capsule Delayed inhibitory effects (sleep sleep like states) have been evoked by stimulation of the caudate nucleus at low rates Whether the response was due to spread of current to neighbouring structures has not been investigated The response is non specific it is elicited by stimulation of the diencephalon brain stem, peripheral nerves and receptors

In unanesthetized cats spinal root to root reflexes and spindle afferent discharges are enhanced by stimulation of the caudate nucleus, globus pallidus and nucleus ventralis anterior of thalamus After moderate doses of nembutal, stimulation of the above regions inhibits root to root reflexes and afferent discharges from extensor muscles

Chapter 3

HISTOLOGY

Lesions and stimulation in the different parts of corpus striatum indicate that a discrete function cannot be assigned to any individual nucleus Normal function is the result of interactions within the corpus striatum and between it and other portions of the brain The cells and fibers within the corpus striatum and the pathways that connect corpus striatum with other brain regions are considered in this chapter and the next

A CELLS AND SYNAPSES

The following description is based on *Bielschowsky s* (1919) detailed study of the normal histology of the corpus striatum in man The classical descriptions still provide most of our knowledge of the cytology of the corpus striatum To my knowledge the corpus striatum has not been investigated by electronmicroscopy

The caudate nucleus and putamen have the same structure with cells of two types

1) Most cells are small (8–10 μ) and polymorphic of Golgi type II they are slightly larger in putamen than in the caudate nucleus (*Cajal* 1911) The axons are short with terminal arborizations close to the cell body and the numerous dendrites are short and thin Nissl substance is absent in both cell bodies and dendrites

2) The larger cells are either of Golgi type I with axons that make contact with neurones in other parts of the brain or of Golgi type II with axons forming terminal arborizations close to the cell body In *Cajal's* drawing the diameter of the larger cells is about two times that of the small cells (*Cajal* 1911 fig 325) The axons of the large type II cells arborize farther from their cell bodies than the axons of the small cells Axon hillocks are small or absent. The strongly branched dendrites contain fascicles of neurofibrils and little or no Nissl substance Nissl substance and inclusions of lipoid pigment are scattered in the heavily staining cytoplasm of the cell body The nucleus is often placed eccentrically Cell bodies and dendrites are covered with one or more concentric layers of satellite cells Other glia cells are rare (*Vogt* and *Vogt* 1920)

Most fibers in the caudate nucleus and putamen are unmyelinated they are arranged in a dense network around the cells and, as in the cortex, synaptic structures are not visible

Globus pallidus contains large, multipolar cells all alike, Golgi type I They are 20–50 μ (*Koelliker* 1896), probably the giant cells (cellules geantes, etoilees") of up to 40–50 μ in diameter described by *Cajal (1911) The cell bodies appear spindle shaped or triangular on sections* The axons look like the axons of pyramidal cells in the cortex and motoneurons in the spinal cord with a long axon hillock tapering grad ually to the point where the myelin sheath begins There is no evidence of axon collaterals inside the globus pallidus In a section of globus pallidus the dendrites appear as small spindle shaped ' cells without nuclei Nissl substance is ample in the cell bodies and in the initial por *tions of the dendrites The nucleus is situated in the middle of the cell* body, it stains lightly and has a dense central nucleolus Glial stains in dicate that cell bodies and dendrites are covered with a sheath of a homogenous substance Numerous glia cells are distributed evenly through *out the globus pallidus (Vogt and Vogt 1920)*

Myelinated fibers are numerous and of larger caliber than in the caudate nucleus and putamen Cell bodies and dendrites are covered with ringshaped terminal boutons One fiber often makes contact with the same cell through many terminal boutons

B EARLIER INVESTIGATIONS OF CONNECTIONS

Forel (1877) described the nuclear configurations of the upper brainstem (Haubenregion) and found fibers ascending from the red nucleus to globus pallidus *Ferrier (1876) considered the* corpus striatum a relay between the cortex and the brain stem *Gowers* (1887) interpreted his experience with brains at autopsy to indicate that the corpus striatum had no connections with the cerebral cortex, he believed that cerebellum of the opposite side received *fibers from and projected back* to the corpus striatum Connections between the corpus striatum and the thalamus *were found by Edinger (1911) Projections from the* caudate nucleus to nucleus niger were suggested by *Holmes* (1901) and confirmed by *Edin ger* (1911) *Cajal* (1911) described *axon collaterals to the* corpus stria tum of cortical efferents passing through the nucleus *Wilson* (1914) in a histological study of monkey brains found no direct connections between the corpus striatum and cortex He believed that the corpus striatum as the head ganglion of an independent *motor system received impulses* from the thalamus and projected to the periphery *via* the rubrospinal

tract *Wilson* suggested an influence via thalamus on the corticospinal system

Conclusions about fiber connections appeared abundantly in the clinicopathological literature on the extrapyramidal system but the difficulty of distinguishing between primary lesions and secondary degeneration makes such results uncertain *Wilson*s finding that the corpus striatum has no direct connections with the cerebral cortex was generally confirmed (*Vogt* and *Vogt* 1919 1920 *Riese* 1924 *von Monakow* 1926 *Kodama* 1929) But the *Vogts* stressed that impulses could be exchanged between the cortex and corpus striatum via thalamus Corpus striatum was believed to send impulses to the periphery via the interconnected nuclei of the extrapyramidal system

The suppressor circuit area 4s→caudate nucleus→globus pallidus→thalamus→area 4 proposed by *Dusser de Barenne* and *McCulloch* (1938) inspired anatomists to reinvestigate connections between corpus striatum and cerebral cortex The capsular axon collaterals of *Cajal* (1911) were confirmed (see p 32) and a path from the globus pallidus to cortex *via* nucleus ventralis anterior and lateralis was described (see p 34) The concept of an old motor system topped by the corpus striatum (*Wilson* 1924) was replaced by the concept of the suppressor circuit This concept implied that the corpus striatum and cortex functioned as a unit

In 1942 the connections of the corpus striatum were fully reviewed by *Pape*.

C INVESTIGATIONS OF CONNECTIONS SINCE ABOUT 1940

1 *Fibers within the corpus striatum*

After lesions in the caudate nucleus or putamen in monkey and cat, degenerated myelin sheaths (*Ranson* *Ranson* and *Ranson* 1941 a *Mettler* 1945 b) and axons (*Johnson* and *Clemente* 1959 *Voneida* 1960 *Johnson* 1961 *Szabo* 1962) can be followed into globus pallidus Lateral areas of the caudate nucleus are mainly connected to the lateral segment of globus pallidus and medial areas of the caudate nucleus are mainly connected to the medial segment of globus pallidus both segments of globus pallidus receive fibers from all parts of putamen (*Szabo* 1962)

Globus pallidus does not project to the caudate nucleus or putamen (*Ranson* *Ranson* and *Ranson* 1941 a) the caudate nucleus does not project to putamen (*Voneida* 1960 *Johnson* 1961) and fibers from putamen to the caudate nucleus have not been reported

Fibers from the medial to the lateral segment of the globus pallidus were described in Marchi material from monkey (*Ranson* *Ranson* and

Ranson 1941 a *Laursen* 1955) and in Glees-stained material from man (*Martinez* 1961) Fibers conducting from the lateral to the medial segment (*Kodama* 1929) were denied by the *Ransons* and have not been reported in studies using the Glees or Nauta staining methods for degenerated axons

2 *Connections with the cerebral cortex*

Fibers from cortex to corpus striatum Unmyelinated collaterals from descending fibers in the internal capsule (*Cajal* 1911) were confirmed by *Glees* (1944) using silver impregnation of degenerated fibers after cortical ablations In cat the primary motor areas, part of the somatosensory projection areas and strip areas 2s, 3s and 8s were removed In rabbit the cortical ablations were more extensive Descriptions of the putamen and globus pallidus were not included in *Glees* report In monkeys degenerated axons occurred in the caudate nucleus after lesions in the posterior orbital gyrus Degenerated fibers were not found in putamen and globus pallidus (*Wall Glees* and *Fulton* 1951, Glees stain) Cortical projections to the corpus striatum have been found in Marchi studies In goat frontal dorsolateral cortex projected to the caudate nucleus, putamen and globus pallidus (*Igarasi* 1940) In monkey *Glees* (1945) described projections from area 6 to the globus pallidus and from area 4 to putamen In primates half of the axons from the cortex to corpus striatum came from frontal cortex and one fifth of those fibers from area 4 (*Mettler* 1943) In a later publication *Mettler* (1947) denied connections from area 4 to the corpus striatum but confirmed connections from area 6 to the caudate nucleus and globus pallidus and from area 4s and the caudal part of area 6 to the caudate nucleus, putamen and globus pallidus

Fibers from corpus striatum to cortex Three months after the frontal cortex of monkeys was removed the volume of the caudate nucleus was reduced This finding not reported in detail, was interpreted to indicate a well defined pathway from the caudate nucleus to neocortex (*Mettler Grundfest* and *Hovde* 1952 *Harman Tankard Hovde* and *Mettler* 1954 *Tankard* and *Harman* 1955) On the other hand degenerated caudate cortical axons were absent after lesions in the caudate nucleus (*Voneida* 1960) and *Burandt French* and *Akert* (1962) found no retrograde degeneration in the caudate nucleus of monkeys after bilateral removal of area 6 area 9 or all cortex

There is thus insufficient evidence of a direct striato-cortical pathway An indirect path via globus pallidus and nucleus ventralis anterior and lateralis of the thalamus is well documented in monkey and cat (*Ranson*

Fig 3

A Connections of the corpus striatum Open circles indicate cell bodies L m lemniscus medialis – *B Simplified diagram* indicating that sensory impulses relayed by the brain stem may via the intralaminar thalamic nuclei go through either or both of two arcs one involves the corpus striatum the other the cerebral cortex The cortex projects directly to the caudate nucleus there are no direct connections from from the corpus striatum to cortex An indirect pathway from the globus pallidus to cortex is via nucleus ventralis anterior or lateralis of the thalamus – For simplicity the connections of the putamen are not indicated they are similar to those of the caudate nucleus.

Ranson and *Ranson* 1941 a b *Papez* 1942 *Woodburne Crosby* and *McCotter* 1946 *Glees* 1945 *Hassler* 1949 *Johnson* and *Clemente* 1959 *Nauta* and *Mehler* 1961)

The recent anatomical findings that direct connections from the corpus striatum to cerebral cortex are absent are in accordance with electrophysiological results (*Laursen* 1961 a b)

3 Connections with the thalamus

Fibers from thalamus to corpus striatum The intralaminar and midline groups of thalamic nuclei project to the corpus striatum This was found in a number of species with different anatomical methods (Table 1) and was indicated by studies of pathological material from humans (*Vogt* and *Vogt*

Table 1

Connections from the thalamus to corpus striatum

	Rat		Rabbit	Cat		Monkey
	Nauta	Retrograde degeneration	Retrograde degeneration	Nauta	Marchi	Retrograde degeneration
Centralis medialis paracentralis and centralis lateralis to caudate nucleus		Powell & Cowan (1954) (no description of spatial organization)	Droogleever Fortuyn & Stefens (1951) Stefens & Droogleever Fortuyn (1953) Cowan & Powell (1955)	Nauta & Whitlock (1954)		Powell & Cowan (1956)
Centrum medianum and parafascicularis to putamen	Hiddema & Droogleever Fortuyn (1960)		Droogleever Fortuyn & Stefens (1951) Stefens & Droogleever Fortuyn (1953) Cowan & Powell (1955)	Nauta & Whitlock (1954) Johnson (1961)	Burgi & Bucher (1960) (also to the caudate nucleus)	Mettler (1947) Powell & Cowan (1956)
Centrum medianum to globus pallidus				Nauta & Mehler (1961) Johnson (1961)	Burgi & Bucher (1960)	

1941 *Freeman* and *Watts* 1947 *McLardy* 1948 *Hassler* 1949 *Simma* 1951) The fibers from midline and intralaminar nuclei fan out in an orderly fashion neighbouring points in the thalamus from midline through intralaminar nuclei send fibers to neighbouring points in structures ex tending from infralimbic cortex through nucleus accumbens fundus of the caudate nucleus head of the caudate nucleus putamen and globus pallidus The pattern is clearest in rodents (*Stefens* and *Droogleever Fortuyn* 1953) because their internal capsule is relatively small With development from rat to monkey the midline nuclei with their infra limbic connections decrease in relative volume and the nuclei centrum medianum and parafascicularis increase This phylogenetic shift appears in the corpus striatum as the increased relative size of the putamen (*Harman* and *Carpenter* 1950) and globus pallidus (*von Bonin* and *Shariff* 1951) in primates

The anatomical basis of the diffuse electrocortical effects elicited from the intralaminar nuclei (*Morison Dempsey* and *Morison* 1941) is un known the arousal not the recruiting response is blocked by mesence phalic transection (*Schlag* and *Chaillet* 1963) Direct cortical projections from the intralaminar nuclei could not be demonstrated with the Nauta method in cats (*Nauta* and *Whitlock* 1954) and rats (*Hiddema* and *Droogleever Fortuyn* 1960) the Marchi method in cats (*LeGros Clark* and *Boggon* 1933) or retrograde degeneration in cats (*LeGros Clark* and *Boggon* 1933) rats (*Rose* and *Woolsey* 1949) rabbits (*Stefens* and *Droogleever Fortuyn* 1953) and monkeys (Powell and *Cowan* 1956)

Only *Nashold Hanbery* and *Olszewski* (1955) interpreted retrograde cell changes in the thalamus of cats and monkeys to indicate cortical projections from the intralaminar nuclei whether projections to the corpus striatum were absent or consisted of collaterals from thalamo-cortical fibers could not be determined

In short the midline and intralaminar thalamic nuclei project to the corpus striatum Whether these thalamic nuclei project directly to the cortex is unsettled

Fibers from corpus striatum to thalamus Fibers from the globus pal lidus can be traced through subthalamic regions H and H_1 as a hook shaped bundle ending in the nucleus ventralis anterior and lateralis of the thalamus (for references see p 34) In addition *Nauta* and *Mehler* (1961) found fibers ending in the centrum medianum in both cat and monkey

The caudate nucleus and putamen project to the thalamus indirectly *via* the globus pallidus (see p 33)

Direct fibers from the caudate nucleus to thalamus were reported in Nauta stained material from monkey (*Schulman* and *Auer* 1957) but were

Table 1

Connections from the thalamus to corpus striatum

	Rat		Rabbit	Cat		Monkey
	Nauta	Retrograde degeneration	Retrograde degeneration	Nauta	Marchi	Retrograde degeneration
Centralis medialis paracentralis and centralis lateralis to caudate nucleus		Powell & Cowan (1954) (no description of spatial organization)	Droogleever Fortuyn & Stefens (1951) Stefens & Droogleever Fortuyn (1953) Cowan & Powell (1955)	Nauta & Whitlock (1954)		Powell & Cowan (1956)
Centrum medianum and parafascicularis to putamen	Hiddema & Droogleever Fortuyn (1960)		Droogleever Fortuyn & Stefens (1951) Stefens & Droogleever Fortuyn (1953) Cowan & Powell (1955)	Nauta & Whitlock (1954) Johnson (1961)	Burgi & Bucher (1960) (also to the caudate nucleus)	Mettler (1947) Powell & Cowan (1956)
Centrum medianum to globus pallidus				Nauta & Mehler (1961) Johnson (1961)	Burgi & Bucher (1960)	

terial from man and monkey (*Papez* 1942 *Verhaart* 1950 1952) and after lesions in putamen in monkeys (*Nauta* and *Mehler* 1961 *Szabo* 1962) but were denied in other reports (monkey *Ranson Ranson* and *Ranson* 1941 a cat *Johnson* and *Clemente* 1959)

In summary the caudate nucleus and globus pallidus send unmyelinated fibers to the zona reticulata of nucleus niger Direct fibers from the putamen to nucleus niger are controversial

Fibers from nucleus niger to corpus striatum Fibers from the nucleus niger to globus pallidus described in normal material (*Papez* 1942) were confirmed in Marchi preparations from cat and monkey (*Kimmel* 1942 *Mettler* 1945 b *Ranson* and *Ranson* 1941) The origin of the fibers appeared to be the zona compacta (*Kimmel* 1942 *Mettler* 1945 b) fibers from the lateral part of zona reticulata went to putamen and fibers from the medial part of zona reticulata went to the caudate nucleus (*Mettler* 1945 b) Rosegay (1944) on the other hand found no evidence of nigropallidal fibers He interpreted his findings as indicating a nigrocaudate connection because of retrograde degeneration found in the nucleus niger after caudate lesions It is difficult however to distinguish between transsynaptic and retrograde degeneration (*Jacob* 1957) In summary the presence of nigropallidal connections is well documented The existence of fibers from the nucleus niger to putamen and to caudate nucleus requires confirmation

6 *Connections with the subthalamus*

Nucleus subthalamicus The fasciculus subthalamicus contains fibers which conduct from the globus pallidus to nucleus subthalamicus (*Ranson Ranson* and *Ranson* 1941 a *Glees* 1945 *Mettler* 1945 b *Woodburne Crosby* and *McCotter* 1946 *Whittier* and *Mettler* 1949 *Verhaart* 1950 1952 *Laursen* 1955 *Johnson* and *Clemente* 1959 *Nauta* and *Mehler* 1961) as well as fibers which conduct from nucleus subthalamicus to globus pallidus (*Glees* and *Wall* 1946 *Whittier* and *Mettler* 1949)

Zona incerta Pallido-incertal fibers (*Kodama* 1929) were confirmed after lesions in the globus pallidus in cat and monkey (*Woodburne Crosby* and *McCotter* 1946 *Laursen* 1955 *Johnson* and *Clemente* 1959)

7 *Connections with the mesencephalic tegmentum including the red nucleus*

Fibers from corpus striatum to mesencephalon In Marchi preparations from monkey *Ranson* and *Ranson* (1941) found no evidence of pallidal efferents beyond the mesencephalon Investigation of the normal brain

not confirmed in cat and monkey (*Voneida* 1960, *Johnson* 1961, *Nauta* and *Mehler 1961*, *Szabo 1962)* In rat some evidence was found of fibers from putamen to nuclei parafascicularis and reticularis (*Hiddema* and *Droogleever Fortuyn* 1960) Direct fibers from the caudate nucleus or putamen to thalamus *via* globus pallidus are unlikely because fibers of the unmyelinated type characteristic of the caudate nucleus and putamen were *not found in fields of Forel H* and H_1 *(Verhaart 1950)*

4 *Connections with the hypothalamus*

Such connections were described in older studies (for references see *Papez* 1942) A well defined pallido-hypothalamic tract can be traced to the ventromedial nucleus of the ipsilateral hypothalamus (*Vidal 1940, Ran son Ranson* and *Ranson* 1941 a, *Glees* 1944, 1945, *Mettler* 1945 b, *Laur sen* 1955, *Johnson* and *Clemente* 1959) Furthermore efferent fibers from the globus pallidus have been traced to the supraoptic nucleus (*Mettler 1945* b) the periventricular grey substance (*Woodburne Crosby* and *Mc Cotter* 1946) and the periformical region (*Johnson* and *Clemente* 1959) The origin of the tract to hypothalamus was the globus pallidus *Glees* (1944) in addition found fibers which had their origin in the contralat eral putamen This ' striohypothalamic tract crossed the midline as a *part of Ganser s commissure*

5 *Connections with the nucleus niger*

The polarity and origin of fibers which connect the corpus striatum with the nucleus niger (the comb bundle *) has been debated Studies of fiber* degeneration after experimental lesions indicate that fibers pass between the two structures in both directions

Fibers from corpus striatum to nucleus niger In Nauta stained material *from cat and monkey fibers from* the caudate nucleus or putamen tra versed the internal capsule and basis pedunculi to end in the zona re ticulata of the nucleus niger (*Voneida* 1960 *Johnson* 1961 *Nauta* and *Mehler* 1961 *Szabo 1962) Fibers from the* globus pallidus to zona re ticulata of nucleus niger (for references see *Papez* 1942) were denied on the basis of Marchi material from monkey (*Ranson Ranson* and *Ran son* 1941 a) and in normal material (*Verhaart 1950) but* pallidonigral connections were confirmed in material from cat stained with the Nauta *method (Johnson* and *Clemente* 1959 *Nauta* and *Mehler* 1961) Direct fibers from the putamen to nucleus niger were described in normal ma

exist to structures in the rhombencephalon and in the spinal cord The zona incerta nucleus niger tegmentum of the midbrain and inferior olivary nucleus (*Pape* 1942 *Woodburne Crosby* and *McCotter* 1946) as well as nucleus reticularis tegmenti ventralis and nucleus precursorius of Ziehen (*Ogawa* 1952) were proposed as relays from the corpus striatum to cerebellum

Fibers from the lower brain stem to corpus striatum Fibers from the medial lemniscus to the globus pallidus (*Spitzer* and *Karplus* 1907) were confirmed by *Glees* (1944) The fibers crossed the midline in the ventral part of Ganser s commissure In pathological material *Hassler* (1949) found fibers ascending to the globus pallidus from medial lemniscus spinothalamic tracts and interstitial nucleus

SUMMARY

The main source of fibers to the corpus striatum is the intralaminar nuclei of the thalamus These fibers transmit impulses from the sensory relay nuclei of the thalamus and from the reticular formation of mesencephalon and rhombencephalon Other fibers to the corpus striatum arise in the cerebral cortex and in the mesencephalon particularly in nucleus niger

Corpus striatum sends fibers to nucleus ventralis anterior and lateralis and possibly to centrum medianum of the thalamus Furthermore the corpus striatum projects to hypothalamus nucleus subthalamicus zona incerta nucleus niger nucleus ruber and the midbrain tegmentum By way of these connections the corpus striatum indirectly projects to all structures of mesencephalon and rhombencephalon and to the spinal cord The corpus striatum and cerebral cortex form two internuncial arcs between the intralaminar nuclear group of thalamus and the descending tracts of the brain stem (fig 3) The cortex and corpus striatum may be looked upon as a) internuncial systems coordinating the activity of the thalamic nuclei and b) the only significant pathways from the thalamus to the efferent system (*McCulloch* 1944)

of a Sumatra gibbon disclosed no pallidal fibers to the red nucleus (*Verhaart* 1950 1952) In a study of normal material and some Marchi preparations from monkey *Woodburne Crosby* and *McCotter* (1946) found fibers from the corpus striatum to the midbrain tegmentum *Laursen* (1955) in Marchi material from monkey traced fibers from the fasciculus lenticularis to the dorsomedial part of the red nucleus and to the tegmentum dorsal to the red nucleus (nucleus mesencephalicus profundus pars dorsalis) These *mesencephalic connections were confirmed in Marchi* (*Burgi* and *Bucher* 1960) and Nauta stained cats brains (*Johnson* and *Clemente* 1959) In addition terminal degeneration occurred in the nucleus interstitialis of the medial longitudinal fasciculus the nucleus of Darkschewitsch and in other parts of the periventricular grey substance (*Johnson* and *Clemente* 1959)

In two human brains stained with the Glees silver technique 4 and 17 days after pallidectomy terminal degeneration was found in the red nucleus and adjacent tegmentum (*Martinez* 1961)

In summary studies using the Marchi the Nauta and the Glees techniques indicate direct pallidal efferents to the mesencephalon In addition indirect routes are available *via* synapses in the field H of Forel zona incerta, nucleus subthalamicus and nucleus niger (see p 38)

Fibers from mesencephalon to corpus striatum Fibers ascending from the red nucleus to globus pallidus were described in Marchi material from monkey (*Carpenter* 1956) and in Nauta stained material from cat (*Johnson* and *Clemente* 1959, *Hinman* and *Carpenter* 1959) The connec*tion arose from both rostral and caudal halves of the red nucleus* and was mainly uncrossed (*Carpenter* 1956) In addition direct projections to the corpus striatum originated in the rostral midbrain tegmentun near the red nucleus (*Nauta* and *Kuypers* 1958 *Burgi* and *Bucher* 1960) In Golgi stained material from young mice some reticulothalamic axons went be vond the reticular nucleus of the thalamus, they appeared to continue into corpus striatum (*Scheibel* and *Scheibel* 1958)

8 *Connections with the brain stem below mesencephalon*

Fibers from corpus striatum to the lower brain stem Lesions in the caudate nucleus and globus pallidus produced terminal degeneration in the inferior olive (Glees and Nauta stains *Walberg* 1956) That all lesions extended into the internal capsule was believed not to invalidate the results because the author *assumed* that cortex and corpus striatum project to separate regions of the inferior olive *Other* direct *fibers to the lower brain* stem (*Morgan* 1927) have not been confirmed Indirect routes

exist to structures in the rhombencephalon and in the spinal cord The zona incerta nucleus niger tegmentum of the midbrain and inferior olivary nucleus (*Pape*, 1942 *Woodburne Crosby* and *McCotter* 1946) as well as nucleus reticularis tegmenti ventralis and nucleus precursorius of Ziehen (*Ogawa* 1952) were proposed as relays from the corpus striatum to cerebellum

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Chapter 4

ELECTRICAL SIGNS OF CONNECTIONS BETWEEN THE CORPUS STRIATUM AND OTHER PARTS OF THE BRAIN

A STIMULATION OF CORPUS STRIATUM

1 *Effect on spontaneous cortical activity (electrocortical activation)*

Repetitive stimulation of the caudate nucleus has been reported to decrease the amplitude and to increase the frequency of the electrocorticogram (*Morison Dempsey* and *Morison* 1941, *Gerebtzoff* 1941, *Ferro Milone Lusso* and *Terzuolo* 1953 a b, *Stoupel* and *Terzuolo* 1954, *Shimamoto* and *Verzeano* 1954, *Tokizane Kawakami* and *Gellhorn* 1957, *Heuser* et al 1961) *Starzl Taylor* and *Magoun* (1951) in a study of the diencephalic extension of the reticular activating system stated that the internal capsule rather than the basal ganglia appeared to be the excitable focus *Demetrescu* and *Demetrescu* (1960) claimed that electrocortical activation elicited from the caudate nucleus was due to spread of current to the thalamus

I have used stimulation through the lead pairs of the 14 lead electrode (100–200/sec) to localize the regions in and around the caudate nucleus responsible for electrocortical arousal Stimulation of the reticular nucleus of the thalamus and of the internal capsule evoked arousal arousal was absent when the caudate nucleus was stimulated (Fig 4) The failure to find electrocortical arousal during stimulation of the head of the caudate nucleus was not due to damage produced in it by the multilead electrode i) electrocortical arousal was elicited from the thalamus where the damage produced by the electrode was the same as in the caudate nucleus ii) The absence of electrocortical arousal was confirmed by stimulation between a thin needle electrode and a distant indifferent electrode The needle inflicted a minimum of damage due to the small volume of its pointed tip Stimulation of the reticular nucleus of the thalamus with the same electrodes elicited electrocortical arousal Stimulation in the center of the head of the caudate nucleus at the same strength and at a strength up to three times the threshold found in the thalamus did not evoke electrocortical arousal

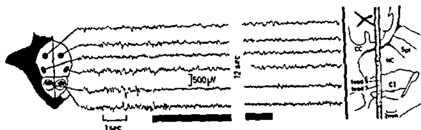


Fig 4

Electrocortical arousal produced by stimulation of the right internal capsule through the multielectrode (electrode pair 8–9 1 msec pulses at 200 sec indicated by black line) – An identical response was elicited by stimulation through the electrode pair 7–8 Stimulation through other adjacent electrode pairs did not elicit electrocortical arousal Cat, encephale isole Extradural unipolar recording Recording sites shown to the left stimulation ites to the right. The strength of stimulation was 2 5 times the threshold for ipsilateral pericruciate arousal The flattening outlasted the stimulus about 5 sec and was most pronounced in the ipsilateral pericruciate region slightly less pronounc d in the contralateral pericruciate region and just detectable in the other lead. – CC Corpus callosum CI Capsula interna. NC Nucleus caudatus Spt Area septalis (*Laursen* 1961 b)

Thus electrocortical activation evoked from the caudate nucleus may be ascribed to spread of current from the commonly used stimulating electrodes There is no evidence to support the view expressed by *Jung* and *Hassler* (1960) that the caudate nucleus is a part of or closely related to the ascending reticular activating system

After stimulation of the caudate nucleus (120/sec) the electrical activity recorded from the hippocampus was increased in amplitude and the pre dominant frequency shifted from 2 5 to 4/sec (*Tokizane Kawakami* and *Gellhorn* 1959) The possible role of spread of current to the internal capsule or thalamus was not investigated During arousal in neocortex associated with behavioral alertness waves of high amplitude can be re corded from the hippocampus and the predominant frequency shifts from 3–6/sec to 5–7/sec (*Green* and *Arduini* 1954)

In monkeys observed for two years unilateral and bilateral lesions in the caudate nucleus in putamen or in both produced hypersynchrony of brain waves bursts of 5–10/sec waves of large amplitude appeared in all leads and 15–20/sec activity became rare or disappeared When ablation of areas 4 or 6 was added to the lesions in corpus striatum the cortical electrical changes were more pronounced (*Kennard* and *Nims* 1942)

2 *Gross responses in the cerebral cortex*

The effect of electrical stimulation of the caudate nucleus depends on the frequency of stimulation

a *Short latency responses* In cats single shocks are reported to elicit cortical responses of short latency (*Jung* and *Tonnies* 1950 *Wieck* et al 1960), *interpreted to indicate transmission to the cortex by a direct* pathway (*Shimamoto* and *Verzeano* 1954, *Purpura Hausepian* and *Grundfest* 1958) *Purpura* et al believed that the stimulating current did not spread to the internal capsule because no potential change was recorded in the medullary pyramid However, the threshold of cortical afferents in the internal capsule may be lower than that of efferents *Stoupel* and *Terzuolo* (1954) showed that the threshold of the short latency cortical responses was lower in the internal capsule than in the caudate nucleus and this indicates activation of cortical afferents in the internal capsule (*Stoupel* and *Terzuolo* 1954) However these workers used thyratron generated stimuli and the thresholds were given in arbitrary units Therefore *the observed differences in threshold may have been due to differ*ences in impedance even with the same electrode inserted successively *in the caudate nucleus and internal capsule*

To exclude this possible source of error I have measured both stimulating current and stimulating voltage Threshold currents were calculated from the voltage drop over a ten ohm resistor in series with the stimulating leads and recorded on one beam of a two beam oscilloscope The voltage was displayed on the other beam To reduce artefact, the floating output of the stimulator was isolated from the amplifier used to measure threshold currents by a double screened transformer (*Buchthal*, *Guld* and *Rosenfalck* 1955, *Guld* 1961 Fig 5)[4]) With one msec rectangular pulses the impedance $\left(\left|\frac{V}{I}\right|\right)$ of adjacent lead pairs of the electrode was 20–30 kohms, with no systematic difference between the caudate nucleus and internal capsule In this way *Stoupel* and *Terzuolo*'s result was confirmed that the threshold of short latency cortical responses was higher in the caudate nucleus than in the internal capsule (Fig s 6 7) The threshold in the internal capsule was as low as 0 1 mA in the caudate nucleus it was up to 20 times higher Similarly a larger cortical response was evoked from the internal capsule and thalamus than from the caudate nucleus (*Laursen* 1961 a) The complex potentials recorded from the cortex after caudate stimulation by *Purpura Hausepian* and *Grundfest* (1958) outlasted the cortical response to stimulation of the internal capsule spread of the stimulus to thalamus or corpus callosum may explain this finding

[4]) The isolation transformer is not necessary if the stimulating current is measured with a difference amplifier with high common mode rejection at the stimulating voltage Conventional difference amplifiers however do not sufficiently reject more than one volt

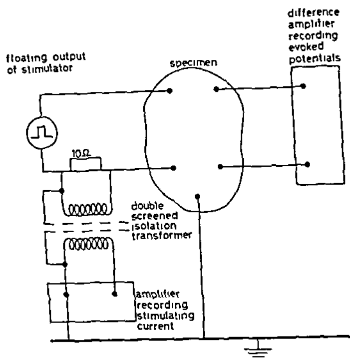


Fig 5

Recording of evoked potentials with measurement of stimulating current and voltage To reduce artefact the output of the stimulator is made to float with respect to ground (*Guld* 1961) The stimulating current is measured *via* a double screened transformer The stimulating voltage is indicated on the dial of the stimulator

The results of *Stoupel* and *Terzuolo* (1954) and *Laursen* (1961 a) in dicate that stimulation confined to the caudate nucleus does not evoke a cortical response This is in agreement with recent anatomical evidence that there are no direct caudate-cortical fibers (*Voneida* 1960 *Burandt French* and *Akert* 1962) and with the absence of spikes in recordings from the cortex after injection of strychnine into the caudate nucleus (*Dusser de Barenne* and *McCulloch* 1938 *Dusser de Barenne Garol* and *McCulloch* 1942 *Garol* and *McCulloch* 1944)

In a recent note *Goldring* et al (1963) compared caudate stimulation in cat and monkey Short latency responses were evoked in the cortex of cats but were absent in monkeys In monkeys thalamocortical fibers to the sensorimotor cortex lie more caudally than in cats and at a greater distance from the caudate nucleus Therefore the authors attributed the results in cats to spread of current to the internal capsule This con firms the findings of *Stoupel* and *Terzuolo* (1954) and *Laursen* (1961 a)

Stimulation of the caudate nucleus may evoke a potential at the site

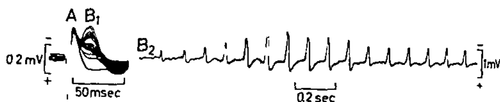


Fig 6

Cortical responses to stimulation in the region of the caudate nucleus (cat chloralose anesthesia 80 mg/kg) – Fifteen superimposed traces of the short latency response (A) and recruiting response (B_1) elicited in ipsilateral anterior sigmoid gyrus Stimulation through an electrode pair in the lateral part of the caudate nucleus (*Laursen* 1961 a) The thresholds indicate that both responses are elicited by spread of current to the internal capsule (Fig 7) A continously recorded recruiting response (B_2) elicited from the thalamus is shown for comparison

of the stimulating electrodes (p 54) and this potential may be recorded from the perisigmoid cortex with a distant electrode used as reference (*Laursen* 1961 a)

b *Evoked spindles and recruiting responses* In a recruiting response (Fig 6) each potential is preceded by a stimulus (1–15/sec) whereas in a "spindle response" the whole series of potentials is evoked by a single shock The following findings indicate that recruiting responses and evoked or spontaneous spindles have their origin in the same functional system in the brain (*Morison Dempsey* and *Morison* 1941 *Morison* and *Dempsey* 1942)

1) Both recruiting responses and spindles are elicited from intralaminar thalamic nuclei
2) Both can be recorded from widespread cortical areas
3) Individual potentials in a recruiting series are similar to individual potentials of a spindle with a large negative and a small positive phase
4) Waxing and waning of recruiting re ponses is similar to the smooth increase and decrease in the amplitude of a spindle
5) Phase reversal of recruiting responses and of spindles often occurs at the same depth in the cortex (*Li Cullen* and *Jasper* 1956)

Many workers have reported that stimulation of the caudate nucleus evokes cortical recruiting responses (*Stoupel* and *Terzuolo* 1954 *Umbach* 1959 *Demetrescu* and *Demetrescu* 1960) and spindles (*Jung* and *Tonnies* 1950, *Shimamoto* and *Verzeano* 1954, *Stoupel* and *Terzuolo* 1954 *Tokizane Kawakami* and *Gellhorn* 1957 *Umbach* 1959, *Wieck* et al 1960 *Demetrescu* and *Demetrescu* 1960 *Buchwald* et al 1961 a) *Umbach* (1959) used stimulating currents of 2 5–25 mA In my experiments with the multielectrode the threshold of cortical recruiting responses was low (0 04–0 1 mA) when elicited from the internal capsule and 10–20 times higher when elicited from the head of the caudate nucleus (Fig 7) Similarly cortical recruiting responses were larger when elicited by stimulation through electrode pairs in the internal capsule and thalamus

(*Laursen* 1961 a) than when elicited through electrode pairs in the caudate nucleus Thus recruiting responses too are elicited by spread of current to the internal capsule rather than by stimulation of the caudate nucleus

Heuser et al (1961) and *Buchwald* et al (1961 b) assumed that afferent impulses upon reaching the thalamus pass to the caudate nucleus and back to thalamus before they proceed to cortex (caudate loop) This hypothesis is based on the assumption that activation of the caudate nucleus may evoke cortical spindles *via* thalamus My attempts to determine the true site from which cortical spindles are triggered when stimuli are applied through an electrode in the caudate nucleus have not been successful Even in Nembutal ® induced sleep the threshold of spindles varied so much that thresholds to stimulation in the caudate nucleus and in adjacent structures could not be compared *Tokizane Kawakami* and *Gellhorn* (1957) and *Buchwald* et al (1961 d) did not have this difficulty in awake paralyzed cats (Intocostrin® Flaxedil®))

After lesions in nucleus ventralis anterior of the thalamus cortical spindle responses could not be evoked from the caudate nucleus (*Shimamoto* and *Verzeano* 1954 *Buchwald* et al 1961 d) To the authors this finding indicated a pathway from caudate nucleus to cortex via nucleus ventralis anterior of the thalamus an alternative explanation is that cortical spindles were elicited by spread of current from the caudate nucleus to nucleus ventralis anterior Cortical spindles were abolished after a metal hook had been pulled between the head of the caudate nucleus and the thalamus (*Heuser et al* 1961) and this too was interpreted to indicate a pathway from the caudate nucleus to cortex *via* thalamus In my opinion the hook may have injured those projections which take a rostral course from nucleus ventralis anterior through the internal capsule to cortex (*Nashold Hanbery* and *Olszewski* 1955)

In monkeys caudate stimulation did not evoke cortical recruiting responses (*Goldring* et al 1963) For the interpretation of this result see p 45

After injection of physostigmine into the caudate nucleus stimulation of this nucleus did not elicit cortical spindle responses but generalized convulsions (*Rakic Buchwald* and *Wyers* 1962) This experiment is difficult to interpret because the diffusion of physostigmine from the site of injection cannot be predicted and because small doses of acetylcholine have an excitatory effect larger doses a blocking effect (*Buchthal* and *Lindhard* 1937)

Lesions in the globus pallidus did not abolish cortical recruiting re

*) Cortical recruiting responses were evoked by stimulation through an electrode situated in the caudate nucleus in man Spindle responses were absent Histological verification was not, of course possible (*Housepian* and *Purpura* 1963 p 76)

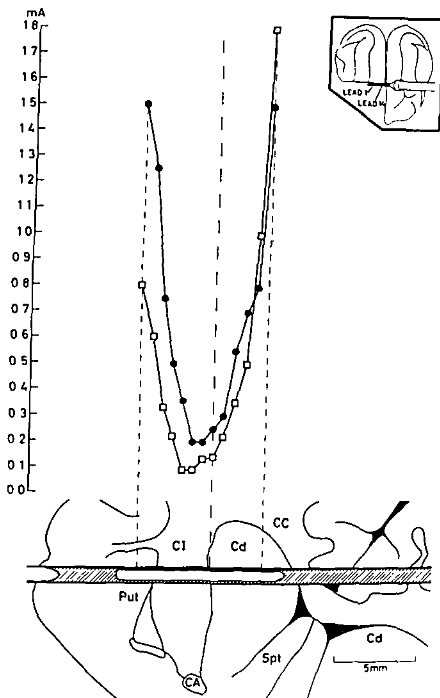
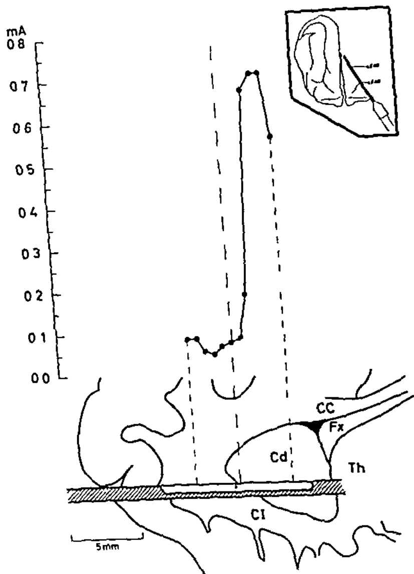


Fig 7

Thresholds of cortical responses to stimulation of the caudate nucleus and internal capsule (Cat Nembutal ® 40 mg/kg) – Stimulation through different adjacent elec trode pairs of a multielectrode (1 msec pulses at 7/sec) elicited a short latency re sponse (Fig 6 A) and a recruiting response (Fig 6 B_1) in the ipsilateral anterior



sigmoid gyrus The thresholds (in mA s) of the short latency response (open squares) and of the recruiting response (filled circles) were about 20 times higher *in the middle* of the caudate nucleus (Cd) than in the internal capsule (CI) The short latency response was elicited by excitation of the specific the recruiting response by excitation of the unspecific cortical afferents in the *internal capsule* – A and B represent brains with different planes of insertion of the multielectrode (ee inset) – CC *Corpus callosum* Fx Fornix, Put Putamen Spt Area septalis, Th Thalamus

sponses elicited by stimulation of the thalamus (*Szekely* 1957) This result is to be expected (see p 37)

3 *Facilitation and inhibition of cortical responses*

Several workers have stimulated the caudate nucleus immediately before or together with another region Depending on the experimental conditions caudate stimulation increases or decreases evoked potentials in the cerebral cortex of cats

a A conditioning shock to the caudate nucleus in *alert animals* increased the amplitude of the cortical response to a flash of light or to electrical stimulation along the visual pathway when the interval between the stimuli was 50 to 200 msec Nembutal© (6 mg/kg) or chloralose (50 mg/kg) abolished the effect (*Buchwald* et al 1962, *Fox* and *O Brien* 1962), at intervals of 20 to 50 msec the amplitude of the cortical response to a flash of light or a thalamic stimulus was decreased (centrum medianum, *Fox* and *O Brien* 1962)

b In *cerveau isole* electrocortical activation elicited by stimulation of nucleus ventralis anterior of thalamus was prevented or abreviated by caudate stimulation of low intensity (100/sec) Caudate stimulation reduced cortical responses to stimulation of thalamic sensory relay nuclei

c In barbiturate *anesthesia* cortical responses increased during the first one to three seconds of caudate stimulation then decreased After transection of the mesencephalon evoked cortical responses were decreased in the beginning of caudate stimulation as well (*Demetrescu* and *Demetrescu* 1960 1961, 1962) In chloralose anesthesia stimulation of the corpus striatum internal capsule and corpus callosum reduced cortical responses to stimulation of the contralateral foreleg, the threshold of this effect was lower in the internal capsule than in the caudate nucleus (Fig 8, *Krauthamer* and *Albe Fessard* 1961)

In brief, evoked cortical responses were increased by caudate stimulation in intact alert animals in anesthetized animals and after transection of the mesencephalon caudate stimulation reduced evoked cortical responses

These results have been interpreted to indicate a facilitatory or an inhibitory action of the caudate nucleus on neurons in the cortex However there is evidence that the amplitude of evoked cortical potentials is not a measure of diffuse cortical facilitation or inhibition Electrocortical arousal increases the cortical response to stimulation of thalamic sensory relay nuclei and decreases the cortical response to stimulation of receptors (*Gauthier Parma* and *Zanchetti* 1956 *Dumont* and *Dell* 1958, *Bremer* and *Stoupel* 1959)

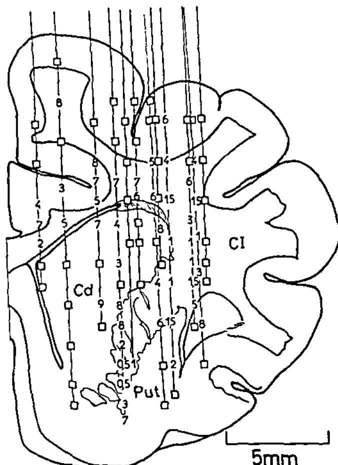


Fig 8

Thresholds of inhibition of cortical responses elicited by sensory stimulation (Krauthamer and *Albe Fessard* 1961 cats anesthetized with chloralose) – Potentials evoked in the posterior suprasylvian gyrus by stimulation of a contralateral leg were diminished by subcortical trains of stimuli (0 5 msec pulses at 200 sec for 25 msec) The numbers (threshold in volts) or an open square (ineffective site or a threshold of more than 8 volts) indicate sites of stimulation Single shocks did not diminish the cortical response Cd Nucleus caudatus CI Internal capsule Put Putamen

Krauthamer and *Albe Fessard* (1961) found lower thresholds of cortical inhibition in the internal capsule than in the caudate nucleus This is consistent with my interpretation that the inhibition was elicited by spread of current to specific or non specific cortical afferents in the internal capsule

4 *Inhibition of electrical seizure activity*

Stimulation of the caudate nucleus with single shocks of moderate intensity reduced the frequency of continuous seizure discharges in the cortex (*Umbach* 1959), high intensity shocks and repetitive stimulation were less effective This was believed to be due either to blocking of the caudate nucleus or activation of adjacent structures by current spread Even the weakest stimulating currents used in these experiments (2 5 mA) are sufficient to activate adjacent structures

5 *Single unit responses in the cerebral cortex*

Single shocks and repetitive stimulation (3–100/sec) of the caudate nucleus inhibited the spontaneous activity of 97 out of 101 single units in the motor cortex of cats prepared as encephale isole Inhibition often out lasted stimulation and was elicited from the caudate nuclei of both sides Only 10 out of 78 units discharges in response to stimulation of the ipsilateral caudate nucleus none responded to contralateral stimulation Signs of spread of stimulating current to the internal capsule such as a short latency gross potential in cortex or evoked activity in the medullary pyramids were absent (*Spehlman Creutzfeldt* and *Jung* 1960) Single units in the visual cortex were inhibited by single shocks to the caudate nucleus but discharged by 50 or 100/sec stimulation (*Lehmann Koukkou* and *Spehlmann* 1962) The intracellularly recorded spontaneous action potentials of 54 cortical interneurons and Betz cells were inhibited by caudate stimuli separated more than 300 msec this was accompanied by hyperpolarization of the cells (*Klee* and *Lux* 1962) The possibility of spread of current to the thalamus was not considered in these studies

6 *Effects on subcortical activity*

A multitude of responses have been described by different workers Stimulation by single shocks with an electrode in the thalamus of cats (Nembutal® *Shimamoto* and *Verzeano* 1954 encephale isole *Umbach* 1959 Flaxedil® *Buchwald* et al 1961 d) With stimulation at 4–8/sec recruiting responses were observed in intralaminar thalamic nuclei (*Umbach* 1959) and Fernentladungen (possibly recruiting responses) in medial thalamus and cornu Ammonis (Pernocton® or Dial® *Jung* and *Tonnies* 1950) In the entopeduncular nucleus subthalamic nucleus substantia nigra and midbrain biphasic waves appeared after a latency of about 6 msec (*Shimamoto* and *Verzeano* 1954) On the other hand *Mettler Grundfest* and *Hovde* (1952) and *Hovde* and *Mettler* (1953) reported

in brief notes that single shocks delivered to the caudate nucleus and putamen evoked short latency spikes or large amplitude activity lasting up to 100 msec or both in different subcortical structures (internal capsule globus pallidus ansa and fasciculus lenticularis thalamus substantia nigra red nucleus tectum and tegmentum) Responses in different subcortical nuclei were also found by *Ferro Milone Foresto* and *Lusso* (1953) *Ferro Milone Lusso* and *Terzuolo* (1953 b nembutalized cats) *Buchwald* and *Ervin* (1957 alert cats) *Szabo* and *Auer* (1958 cats and monkeys) and *Levy Monnier* and *Krupp* (1959 alert rabbits)

The potential recorded from the medullary pyramid of cats was reduced by caudate stimulation (*Buser Encabo* and *Borenstein* 1961) A possible interpretation of this result is that fibers in the internal capsule were activated by current spread and that reduced pyramidal discharge is associated with the arrest reaction (p 26 *Laursen* 1962 b) In a short note *Wieck Buchwald* and *Wyers* (1959) reported that responses in the cortex and midbrain evoked by stimulation of globus pallidus were inhibited or facilitated by a preceding caudate stimulus depending on the length of the interval between stimuli Cortical and midbrain responses to a caudate stimulus were not changed by a preceding stimulus to globus pallidus

The above experiments were thought to indicate anatomical and functional interrelations between the different structures However precautions to detect the effect of spread of stimulating current or of action currents were not reported

B RECORDING FROM CORPUS STRIATUM

1 Spontaneous activity

In encephale isole cats the electrical activity recorded from the caudate nucleus and cortex with gross electrodes had the same frequency (*Stoupel* and *Terzuolo* 1954) In rabbit and cat spontaneous spindles recorded from the caudate nucleus appeared concomitantly with spindles recorded from frontal cortex (*Jung* and *Kornmuller* 1939 *Stoupel* and *Terzuolo* 1945) In unanesthetized rabbits loud noises reduced the amplitude of activity recorded from the caudate nucleus (*Jung* and *Kornmuller* 1939) Spontaneous electrical activity in the caudate nucleus was also depressed when a motor response to sound was established by applying a constant current to the cortical motor area (rabbit *Naumova* 1957) In Dial® anesthesia bursts of large spikes appeared in the caudate nucleus in acute and chronic decorticate monkeys (*Kennard* 1943) and in chronic decorticate cats (*Morison* and *Basset* 1945)

Attempts have been made to identify subcortical structures by the electrical activity recorded with stereotactically inserted electrodes In cats awake and anesthetized with nitrous oxide, *Jung* and *Kornmuller* (1939) recorded faster activity from the caudate nucleus than from lateral thalamus In cats anesthetized with chloralose small but statistically significant differences in the mean amplitude of the spontaneous activity were found in the entopeduncular nucleus ansa lenticularis and optic tract, these differences were absent when Nembutal® was the anesthetic (*Levine* et al 1958) The spontaneous activity of the corpus striatum recorded with depth electrodes in awake humans was similar to that of surrounding structures though the predominant frequency in the putamen and globus pallidus was higher (11–14/sec) than in cortex (*Haynes Meyers* and *Knott* 1949, *Hassler* et al 1960) Cross correlation analysis showed that activity in the corpus striatum preceded that in cerebral cortex (*Brazier* and *Barlow* 1957)

Mihailovic and *Jankovic* (1961) abolished the electrical activity of the caudate nucleus in cats by intraventricular injection of gamma globulin from rabbits treated with homogenates of caudate tissue from cats Gamma globulin from untreated rabbits had no effect

2 Evoked responses

a *Stimulation in the caudate nucleus* Stimulation of the caudate nucleus (*Umbach* 1959, *Wieck* et al 1961) evoked a positive potential in it with a short latency and a stimulus to peak time of 18 msec The subsequent negative phase was larger and it was followed by a number of inconstant oscillations of low amplitude and a spindle response Stimulation and recording was with concentric electrodes The initial phases of the local caudate response were more resistant to anoxia than cortical potentials evoked by caudate (internal capsule, p 44) stimulation (*Wieck* et al 1961) I have stimulated the caudate nucleus discretely through a stereotactically inserted bipolar electrode (two $100 \times 140\,\mu$ areas 0 5 mm apart) and recorded the evoked potential with the same type of electrode placed in the caudate nucleus at a distance of 2 mm from the stimulating electrode A single deflection of short duration was produced by a rectangular pulse (0 5 msec and 2mA) The stimulus to peak time was 4 msec (Fig 9 D) The difference in shape and duration between this response and that described by *Umbach* (1959) and *Wieck* et al (1961) can be attributed to my use of bipolar recording With paired stimuli of equal strength and intervals of 2 to 10 msec the potential evoked by the second stimulus was facilitated (Fig 9 E) Caudate seizure activity rarely occurred even after strong stimulation of the caudate nucleus (*Umbach* 1959) Potentials elicited in the caudate nucleus by caudate stimulation

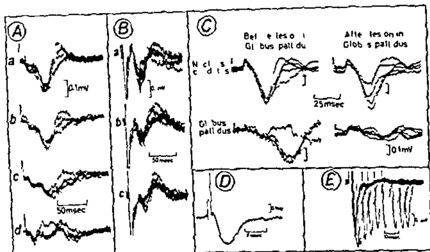


Fig 9

Potentials evoked in the corpus striatum by stimulation of the paws the cortex and the caudate nucleus (cats chloralose 80 mg/kg) – *A* Potentials evoked in the caudate nucleus by electrical stimulation of the ipsilateral forepaw with single shocks (1 msec 10 V) Recording with a concentric electrode. Five uperimposed sweeps The potentials are smaller when the interval between stimuli is reduced The intervals were a 3 sec b 2 sec c 1 sec d 0 5 sec – *B* Recording as in A Stimulation of ipsilateral posterior sigmoid gyrus with rectangular pulses (0 1 msec 20 V) between two steel needles 4 mm apart The first negative and the positive component of the response are smaller the second negative component larger when the interval between stimuli is reduced The intervals were a 3 sec, b 1 sec c 0 5 se – *C* Potentials evoked in the caudate nucleus and in globus pallidus by electrical timulation of the contralateral hindleg Recording as in A A lesion in the globus pallidus did not abolish the potential evoked in the caudate nucleus Therefore the sensory impulses must reach the caudate nucleus via the thalamus (see Fig 3) – *D* Potential evoked in the caudate nucleus by stimulation of the caudate nucleus with a single shock (0 5 msec, 2 mA) Recording and stimulating electrodes were 2 mm apart each was bipolar with 0 5 mm interelectrode distance – *E* Recording and stimulating electrodes as in *D* Paired rectangular stimuli of equal strength two times threshold (2 mA pulse duration 0 5 msec) with different intervals At intervals of 2 to 10 msec the second response is facilitated

spread by volume conduction to pericruciate cortex (*Laursen* 1961 a)

Single units in the globus pallidus discharged when Carbachol® champhor or streptomycin was injected into the caudate nucleus (*Spiegel Wycis Szekely* and *Spuler* 1961) Six of 24 units in alert monkeys discharged in synchrony with a tremor produced by a mesencephalic lesion (*Cordeau* 1961)

b *Stimulation outside the corpus striatum* Spikes were evoked in the globus pallidus when strychnine was applied to dorsal column nuclei in monkey (*Dusser de Barenne Garol* and *McCulloch* 1942) cortical areas 6 4 and 5 in chimpanzee (*Garol* and *McCulloch* 1944) and the

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The results in the literature and my own results are interpreted to indicate that stimulation confined to the caudate nucleus does not change cortical electrical activity The absence of short latency cortical potentials is in agreement with recent anatomical findings that the caudate nucleus does not project directly to the cortex Cortico-caudate projections well documented in anatomical studies have been confirmed using gross and single unit recording from the caudate nucleus

Studies of interactions of the corpus striatum with other subcortical structures cannot be interpreted until the role of spread of current in these experiments has been determined So far there is no evidence to indicate that the caudate nucleus is either a part of or antagonistic to the ascending reticular activating system

thalamic nuclei dorsomedialis and ventralis anterior in cat (*Spiegel Szekely* and *Baker* 1957) Strychnine applied to cortical areas 6 4 and 1 in chimpanzee (*Garol* and *McCulloch* 1944) and posterior orbital gyrus in monkey (*Sachs Brendler* and *Fulton* 1949) evoked spikes in *putamen* From the *caudate nucleus* spikes were recorded when strychnine was applied to areas 8 4s 2 and 24 in chimpanzee (*Garol* and *McCulloch* 1944), posterior orbital gyrus in monkey (*Sachs Brendler* and *Fulton* 1949) and nucleus dorsomedialis of thalamus in cat (*Spiegel Szekely* and *Baker* 1957) Electrical stimulation of the dorsomedial nucleus of the thalamus with single shocks evoked single or multiple waves in the caudate nucleus or in globus pallidus or in both 'Spindles' in these nuclei were most easily elicited by stimulation of nucleus ventralis anterior, when stimulation was repetitive at 4–10/sec recruiting waves appeared (*Jung* and *Tonnies* 1950) Large lesions in the ipsilateral caudate nucleus or ablation of the ipsilateral frontal pole of the hemisphere did not abolish responses in the globus pallidus to stimulation of thalamus (*Spiegel Szekely* and *Baker* 1957) Cortical stimulation evoked gross potentials in the caudate nucleus with latencies so short that a direct pathway seemed to be involved (*Albe Fessard* et al 1960 a *Miranda* 1961) In awake cats immobilized with Flaxedil® stimuli were effective only if delivered at intervals of 15 seconds in chloralose anesthesia however 3 sec intervals were satisfactory I have confirmed this in experiments on cats anesthetized with chloralose (Fig 9 B) I have furthermore confirmed that single units in the head of the caudate nucleus respond to single shock stimulation (2 or 3 sec intervals) of the ipsilateral motor cortex (*Albe Fessard* et al 1960 b) Stimulation at a constant intensity above that which resulted in movement produced one or two spikes (*Laursen* 1961 a) The first spike had a latency of between 15 and 45 msec of the same order as that found by *Albe Fessard* et al (1960 b) who attributed the long delay to synapses within the caudate nucleus

SUMMARY

Electrical and chemical stimulation and recording of electrical potentials may supplement anatomical studies of connections between the corpus striatum and other regions of the brain The use of conditioning and testing stimuli may provide information about complex interactions Unfortunately too few investigators have proved that their results were due to activation of the structure where the electrodes were inserted rather than to spread of current to other structures

1956) In the caudate nucleus too a single unit often responded to peripheral stimuli of different modalities Single units in the caudate nucleus were spontaneously active in cats, awake and anesthetized with Nembutal® but not in cats ane thetized with chloralose Inhibition of spontaneous firing was not elicited by stimulation of receptors Caudate unit responses like gross responses were evoked only by stimuli separated by more than 20 sec chloralose reduced this interval to three seconds (*Albe Fessard* et al 1960 b *Miranda* 1961) Units in the putamen had refractory periods of up to 1700 msec (*Segundo* and *Machne* 1956)

I have explored the caudate nucleus with stainless steel microelectrodes in twelve cats in light nitrous oxide anesthesia The electrodes were in serted through a steel socket chronically implanted in the cat s skull and were advanced by a small motor-driven device (*Andersen* and *Laur sen* 1959) Each cat was used in 1 to 5 experimental sessions and in each session 1 to 5 electrode tracts were explored After the last experi mental session the electrode tip was marked *ad modum Marshall* (1940) Of 60 spontaneously active units 30 discharged at a rate of less than 25/sec 8 at more than 100/sec None responded to touch or electrical stimulation of the skin loud noises or flashes of light Intervals between the stimuli longer than 20 sec as used by *Albe Fessard* et al (1960 a b) were not systematically employed but single stimuli delivered after a pause of several minutes failed to evoke discharges

Chloralose anesthesia abolished spontaneous unit activity in the cau date nucleus and made the units respond to skin stimuli separated by more than three seconds as described by *Albe Fessard* et al (1960 b)

SUMMARY

Gross responses have been reported to occur in all parts of the corpus striatum. They were evoked by different sensory stimuli and in cats immobilized with Flaxedil® or anesthetized with Nembutal® only stimuli separated by more than 20 sec evoked constant responses in chloralose anesthesia 3 sec intervals were sufficient Responses in the caudate nucleus were evoked by clicks at 1/sec if they had been paired repeatedly with shocks to the feet

Unit responses were evoked in all parts of the corpus striatum by dif ferent sensory stimuli in awake paralyzed cats Twenty second intervals between the stimuli were necessary to avoid fatigue of caudate unit responses With units in the putamen in awake cats and with caudate units in cats anesthetized with chloralose the intervals could be reduced to a few seconds In my experiments on cats anesthetized with nitrous oxide responses to sensory stimuli in the caudate nucleus were absent

Chapter 5

SENSORY FUNCTION

Anatomical evidence indicates that impulses from receptors may reach the corpus striatum *via* thalamus or cortex and that globus pallidus receives impulses from the medial lemniscus (p 41, Fig 3)

Gross responses Somatic, visual, auditory and olfactory stimuli evoked gross responses of large amplitude and long latency in the caudate nucleus, putamen and globus pallidus of cats, awake or anesthetized with Nembutal® or chloralose (*Bonvallet Dell* and *Hugelin* 1952, *Albe Fessard* et al 1960 a *Miranda* 1961) *Galambos* (1960) in unrestrained cats found either no or only small, inconstant responses in the caudate nucleus to clicks (1/sec) unless these stimuli had been paired repeatedly with shocks to the feet before the experiment and thus were significant to the animal In *Albe Fessard* et al s experiments only stimuli separated by more than 20 sec from the preceding stimulus evoked responses of constant amplitude in unanesthetized or nembutalized animals In chloralose anesthesia (80 mg/kg) 3 sec intervals were sufficient to avoid fatigue of the responses Section of both the dorsal and the lateral columns in the spinal cord blocked responses whereas section of one or the other column did not In the reticular formation, zona incerta centrum medianum and nucleus ventralis lateralis of the thalamus responses were evoked with shorter latencies than in the caudate nucleus This was interpreted to indicate that impulses went to the caudate nucleus *via* these structures A somatotopic organization of the caudate nucleus was not found (*Albe Fessard* et al 1960 a, *Miranda* 1961)

In six cats anesthetized with chloralose I have confirmed that somatic visual and auditory stimuli evoke gross responses in the caudate nucleus and globus pallidus Stimuli separated by less than 3 sec from the preceding stimulus evoked reduced responses (Fig 9 A) Since an electrolytic lesion in the globus pallidus did not abolish the response the impulses reached the caudate nucleus *via* thalamus (Fig s 3 and 9 C)

Single units in the globus pallidus putamen and claustrum in awake curarized cats responded to peripheral stimuli of different modalities with convergence of two or more modalities on one unit In putamen, in addition, peripheral stimuli inhibited spontaneous firing (*Segundo* and *Machne*

was unaffected by unilateral decerebration (*Rogers* 1922) These findings were confirmed by *Beach* (1952) who used an improved method of observation partially adopted from *Carpenter* (1933) large bilateral lesions in the accessory hyperstriatum and neostriatum eliminated mating behavior After the administration of androgens many birds resumed the copulatory activity and *Beach* concluded that the role of the forebrain in the pigeon s sexual behavior was to maintain a high level of responsiveness to sexual stimuli There is no evidence to indicate that the corpus striatum in mammals has a similar function

B HYPERKINESIA

1 Quantitative recording

Ambulatory hyperkinesia was recorded after frontal lobe lesions (caudate nucleus and cortex) in monkeys (*Richter* and *Hines* 1938) The same effect was produced in monkeys with lesions confined to the frontal areas 8 and 12 (*Kennard Spencer* and *Fountain* 1941) or to area 9 (*French* 1959) *Dean* and *Davis* (1959) reported absence of hyperactivity in a monkey with bilateral lesions of the frontal cortex but cortex in the depth of sulcus principalis was spared

Hyperactivity after lesions confined to the caudate nucleus was observed by *Turner* (1954) *Rosvold* and *Delgado* (1956) *Davis* (1958) and *Dean* and *Davis* (1959) it was denied by *Kennard Spencer* and *Fountain* (1941)

In rats spontaneous activity in an activity cage was unaltered by lesions in corpus striatum (*Beach* 1941) On the other hand *Whittier* and *Orr* (1962) reported an increase in running activity for 30 days after caudate lesions followed by a gradual decrease to preoperative levels

2 General observation

In earlier studies of the effect of discrete lesions in the caudate nucleus in cats and monkeys hyperactivity was not reported (*Wilson* 1914 *Liddell* and *Phillips* 1940) possibly because it may be difficult to detect by simple inspection After lesions confined to the frontal cortex hyperactivity in response to stimuli could be directly observed in monkeys the animals were hyperactive regardless of stimuli after additional lesions in corpus striatum In addition to ambulatory hyperkinesia monkeys with combined lesions pushed obstinately against immovable objects climbed excessively and followed after moving objects in a stereotyped fashion (*Mettler* 1945 a) In summary both lesions of the frontal cortex and of

Chapter 6

BEHAVIORAL STUDIES

A GENERAL OBSERVATION

Behavioral changes were not observed after unilateral and bilateral lesions in the corpus striatum in monkeys (*Wilson* 1914 *Ranson* and *Berry* 1941, *Kennard* 1944) and cats (*Liddell* and *Phillips* 1940) though subtle changes may have gone unnoticed since conditioning techniques were not used

Puppies whose caudate nuclei were removed without ablation of cere bral cortex ran and jumped well but growth was retarded Some animals were agressive and others passive with slow movements not all dogs re sponded to their names though all could find their feeding place and kennels (*Klosovskii* and *Volzhina* 1956)

Comparison of chronic decorticate and decerebrate cats and dogs led *Rioch* (1942) to conclude that none of the reaction patterns which were lost could be localized unequivocally to the corpus striatum On the other hand *Wang* and *Akert* (1962) attributed a number of behavioral changes in high decerebrate cats to damage to the corpus striatum the failure to eat spontaneously to groom themselves and other cats and to engage in sexual activities as well as general hyperactivity agressiveness and increased galvanic skin reflexes The authors did not believe that the severely damaged and de efferented limbic structures could ac count for the observed changes in behavior

Stimulation of the globus pallidus and of the caudate nucleus through implanted electrodes in alert cats and monkeys elicited exploratory be havior feeding and defense reactions eye and ear movements various autonomic responses and inhibition of movements (*Rozhanskii* and *Lagutina* 1957) This diversity of responses suggests activation of several nearby structures (area septalis hypothalamus and amygdala)

The bird provides an opportunity to study the function of a homologue of the corpus striatum in a brain with very little neocortex the bulk of the forebrain corresponds to the mammalian corpus striatum and neo-cortex is represented by only a thin corticoid layer Mating behavior in pigeons was eliminated by bilateral removal of the hyperstriatum and

Nielsen *Doty* and *Ruthledge* 1958 *Brady* and *Conrad* 1960 a globus pallidus in monkeys *Brady* and *Conrad* 1960 a b) In another study a fear response was elicited and the animal could be trained to avoid stimulation (external lamina and posterior part of globus pallidus in monkeys *Delgado* *Rosvold* and *Looney* 1956) That negatively and positively reinforcing regions should exist side by side in the globus pallidus is less likely than that current spread to hypothalamus is responsible for one or the other or for both effects To be positively reinforcing stimulation must be strong (20 to 30 mA *Brady* and *Conrad* 1960 a) and current spread is unavoidable

Bar pressing reinforced with stimulation of the caudate nucleus in cat and globus pallidus in monkey was not suppressed by clicks presented as warning stimuli for a shock to the feet (*Brady* and *Conrad* 1960 a) Bar pressing reinforced with food (without intracranial stimulation) is suppressed by a warning stimulus (*Estes* and *Skinner* 1941) Monkeys trained to press a lever after intervals of 20 seconds since the previous response (5 p 62) to obtain food or stimulation of anterior thalamus most often spaced their reponses only 10 seconds when reinforced with stimulation of globus pallidus This effect is similar to the effect of amphetamine (*Brady* and *Conrad* 1960 b) That globus pallidus is essential for the timing of responses was not confirmed in cats with lesions in globus pallidus performance in pressing a lever at given intervals for food was affected for only 2–3 weeks after the operation (*Hodos* *Laursen* and *Nissen* unpublished results)

3 *Reinforcement by food or avoidance of a shock*

a *Delayed alternation and delayed response* Damage to the frontal lobes in apes and monkeys reduced the number of correct choices in delayed alternation tests (2 p 62) without affecting visual discrimination (*Jacobsen* 1936 *Miles* and *Rosvold* 1956) The same effect and impairment of delayed response learning (3 p 62) was produced both by lesions confined to the frontal cortex and to caudate nucleus (*Rosvold* and *Delgado* 1956 *Rosvold* *Mishkin* and *Szwarcbart* 1958 *Dean* and *Davis* 1959 *Battig* *Rosvold* and *Mishkin* 1960) *Battig* *Rosvold* and *Mishkin* (1962) found impairment of delayed response learning and of visual and auditory discrimination after lesions in the frontal cortex and after lesions in the caudate nucleus The normal performance in visual discrimination tests in previous experiments was ascribed to transfer of the response ("go—no go) from d layed response to visual discrimination learning Stimulation of the caudate nucleus in monkeys had the same effect as lesions delayed alternation was impaired and visual discrimina

caudate nucleus alone and in combination, produce hyperactivity in mon keys

In cats with combined lesions of the caudate nucleus and dorsolateral cortex hyperactivity was observed but it disappeared 10 to 15 days after the operation (*Thompson* 1959)

C CLASSICAL CONDITIONED RESPONSES

In pigeons the frequency of respiration and heart beat increased in response to light or sound stimuli which had been repeatedly paired with shocks to a leg This conditioned response could be established after bilateral removal of the forebrain except the paleostriatum and ecto-striatum Inhibition of responses to light required an intact forebrain (*Tuge* and *Shima* 1959)

D CONDITIONED INSTRUMENTAL RESPONSES

1 *Definitions*

In *instrumental (operant) conditioning* rewards and punishments are made to occur as a consequence of the subject s response or failure to respond The following procedures have been used in investigations of corpus striatum

1) *Avoidance conditioning* The animal must make a response to avoid the stimulus *e g* jump out of a compartment to avoid a shock to the feet or press a lever to interrupt punishing brain stimulation

2) *Delayed alternation* The animal has a choice of two responses and one or the other is rewarded on alternate trials The animal is prevented from making the next re ponse for a certain time (delay) usually 15 seconds The procedure can be varied by reinforcing the same response two (double alternation) or more times before reversal of reward to the other response is made

3) *Delayed response* The animal has a choice of two responses and a stimulus indicates whether one or the other is rewarded When the animal is trained a delay (*e g* 15 seconds) is introduced between stimulus and response During this delay th stimulus is not present

4) *Intracranial self stimulation* Stimulation of many brain areas acts as reward or punishment and may thus reinforce a response If the response (*e g* bar pressing) turns on a rewarding stimulus the animal repeats self stimulation

5) *Spaced responding* A response is only rewarded if a certain time (*e g* 20 se conds) has elapsed since the preceding response If a response is made too early the waiting period starts anew In this way the animal is trained to respond at a low rate

6) *Visual discrimination* The animal must respond differently to different visual stimuli

2 *Reinforcement by intracranial stimulation*

Stimulation of the caudate nucleus did not reinforce intracranial self stim ulation in rats (*Olds* 1956) In cats and monkeys stimulation of the corpus striatum was rewarding (caudate nucleus in cats *Brady* et al 1957

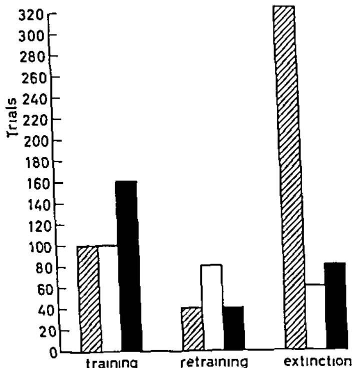


Fig 10

Avoidance learning and extinction in cats with bilateral lesions in the globus pallidus (number of cats in brackets) ▨ Unoperated cats (14) sham operated cats (7 electrode inserted no electrolytic lesion) and control operated cats (10 thalamic lesions) □ Cats with lesions in the globus pallidus trained before and retrained 30 days after the operation (6) ■ Cats with bilateral lesions in globus pallidus trained 30 days and retrained more than 60 days after the operation (6) Extinction occurred more rapidly after lesions in the globus pallidus This indicates reduced fear because the cats performed normally during retraining 60 days after the operation (*Laursen* 1963)

The nucleus niger and globus pallidus are projection areas of the caudate nucleus (*Voneida* 1960 *Szabo* 1962) I placed lesions (*Laursen* 1962 c 1963) in nucleus niger but they damaged adjacent structures and resulted in generalized muscular weakness these cats were unsuitable for behavioral experiments Although lesions in globus pallidus included the lateral part of the genu of the internal capsule motor signs did not appear

After both unilateral and bilateral lesions in globus pallidus conditioned avoidance responses were extinguished more rapidly than in normal and control operated cats This was observed at a time when retention had returned to normal Animals with bilateral lesions in globus pallidus tested 30 days after the operation learned the avoidance response slower than normal and control operated cats Retention tested 30 days after

tion was intact (*Rosvold* and *Delgado* 1956) In four cats, stimulation impaired delayed alternation learning where reversal (see 2 p 62) was made only if the cat chose correctly in 4 out of 5 trials and the delay was 30 seconds In one cat learning was facilitated by caudate stimulation (*Thompson* 1958)

b *Discrimination studies* Rats trained to avoid a shock by choosing an alley sloped upward in favor of one sloped downward lost this ability after bilateral lesions in the globus pallidus (or nucleus ventralis posterior of thalamus) Brain stem lesions had no effect (*Thompson Malin* and *Hawkins* 1961)

Brightness discrimination in rats was unaffected by bilateral lesions in the caudate nucleus or globus pallidus (or hippocampus amygdala and most thalamic nuclei), brightness discrimination was abolished by lesions of thalamus (medial to the geniculate nuclei) of pretectal and of certain hypothalamic nuclei (*Thompson* and *Massopust* 1960) Light dark and flicker frequency discriminations (1 and 10/sec) in cats were unaffected by bilateral stimulation through electrodes in the caudate nucleus provided training to a 100 per cent criterion was completed, during training stimulated cats made more errors than unstimulated cats The stimuli were strong enough to evoke spindle responses in the cortex Furthermore caudate stimulation at 5/sec made the cats hesitate in moving to the food cups (*Buchwald* et al 1961 c) this might be a trace of the arrest reaction fully produced by stimulation at frequencies of 20 to 30 pulses per second (p 26) Cats trained to press a lever to get food decreased their response rate when the caudate nucleus was stimulated at a low frequency when lever pressing was no longer followed by food (extinction) caudate stimulation increased the response rate (*Wyers Buchwald Rakic* and *Lauprecht* 1962) Evaluation of this interesting experiment must await publication of a detailed report

c *Avoidance conditioning* Conditioned avoidance behavior (1 p 62) was investigated in cats with dorsolateral cortical and subsequent caudate lesions (*Thompson* 1959) The added caudate lesions reduced the number of trials to extinction of the avoidance response The number of trials to relearn and the latency of the response were increased when training was completed before caudate lesions were made If caudate lesions were made before training this effect did not appear either because more time had elapsed since the operation or because the lesion damaged mechanisms related to retention of the response

Although the effect on behavior of combined caudate and cortical lesions was absent after isolated cortical lesions it was undecided whether lesions confined to the caudate nucleus could be responsible for the observed change in conditioned avoidance behavior

Chapter 7

BIOCHEMICAL STUDIES

Transmission in central synapses is chemical rather than electrical (*Eccles* 1953 1957) but a central synaptic transmitter has not been identified with certainty Acetylcholine is probably the transmitter between motor axon collaterals and Renshaw cells (*Curtis* and *Eccles* 1958) the membrane of motoneurons is unaffected by acetylcholine (*Eccles Fatt* and *Koketsu* 1954) and another transmitter presumably acts here Whether inhibition is mediated by a special transmitter is uncertain in snail (*Tauc* and *Gerschenfeld* 1960 a) and *Aplysia* (*Tauc* and *Gerschenfeld* 1960 b *Strumwasser* 1962) acetylcholine excites and inhibits different neurons within the same ganglion

Some of the substances mentioned in this chapter may be transmitters in the brain Other substances may continuously regulate neuron excitability as suggested for gamma aminobutyric acid (GABA *Elliott* and *Jasper* 1959) Furthermore the brain and spinal cord contain metabolites which may or may not influence the excitation of neurons

These different substances and the enzymes forming and inactivating them are not equally distributed throughout the brain (for references see *Kety* and *Elkes* 1961) This cannot be explained solely by differences in the relative volume occupied by neuron somata dendrites axons glia or other components Histochemical studies indicate that the composition of neurons differ from region to region (*Gerebtzoff* 1959 *Shute* and *Lewis* 1961) Since the significance of these regional differences is unknown this chapter can be no more than an enumeration of substances and enzyme activities investigated in tissue from the corpus striatum Substances with relatively high concentrations in corpus striatum are mentioned first.

A POSSIBLE TRANSMITTERS

1 Choline acetyltransferase acetylcholine and acetylcholinesterase

The activity of acetylcholine forming enzymes (Acetyl CoA choline O acetyltransferase)[*]) in the caudate nucleus is high exceeded only in the

[*]) Systematic names of enzymes according to the "*Report of the commission on enzymes of the international union of biochemistry*" I U B Symposium series vol 0 Pergamon Press Oxford 1961

bilateral lesions in globus pallidus was impaired as compared with normal and control operated cats, sixty days postoperatively retention was normal (Fig 10) Therefore a memory defect cannot account for the rapid extinction observed more than 60 days postoperatively *Weiskrantz* (1956) and *Waterhouse* (1957) attributed rapid extinction of avoidance responses to diminished anxiety and fear The same interpretation is consistent with the observation in this study that cats with lesions in globus pallidus did not show signs of fear during transfer from the cage to the experimental box, normal and control operated cats vocalized struggled to escape, defecated etc

The effect of electrical stimulation or of crystalline Carbachol® placed in the caudate nucleus, on conditioned avoidance behavior was inconsistent three of seven cats were inattentive to the sound of the buzzer, two failed to cross the barrier and one sometimes raised a forepaw as though about to go through the doorway, but then stopped, looked about and failed to cross (*Stevens Kim* and *MacLean* 1961)

SUMMARY

Behavioral changes in cats and monkeys with lesions confined to the corpus striatum have been observed only when conditioning procedures were used Marked behavioral changes occur in studies comparing decorticate with decerebrate animals It is difficult however, to attribute certain of these changes to damage to the corpus striatum widespread degeneration occurs after extensive multilation of the brain and some of the accompanying behavioral changes are non specific

Quantitative assessment of the spontaneous activity of monkeys and rats under standardized conditions shows that lesions confined to the caudate nucleus or to frontal cortex or to both produce hyperkinesia

Stimulation through electrodes implanted in the corpus striatum in cats and monkeys acts as reward and may reinforce a response But only strong stimulation is reinforcing and spread of current is therefore unavoidable

Performance of delayed responses is impaired after lesions and during stimulation of the caudate nucleus in monkeys and during stimulation of the caudate nucleus in cats Defects in recent memory occur also after lesions in the frontal neocortex Extinction of a conditioned avoidance response in cats is rapid after combined caudate and cortical lesions and after lesions in globus pallidus This can be observed two months after lesions are made in the globus pallidus when a memory defect can no longer be detected The rapid extinction is interpreted to indicate reduced fear of the warning stimulus for the shock

forming dopamin from DOPA (3 4 dihydroxy L phenylalanine) has a high activity in the caudate nucleus (*Holtz* and *Westermann* 1956 *Kuntzman Shore Bogdanski and Brodie* 1961) Unlike dopamin DOPA passes the blood brain barrier and is evenly distributed in grey matter (*Sano* et al 1959)

Dopamin β oxidase transforming dopamin to noradrenaline has a high activity in the caudate nucleus and hypothalamus (*Udenfriend* and *Creveling* 1959) The caudate nucleus contains more dopamin and less noradrenaline and adrenaline than the hypothalamus (*Vogt* 1954) Therefore apart from being a precursor of noradrenaline dopamin in the caudate nucleus and putamen may have another function

Dopamin noradrenaline and adrenaline are inactivated by O-methylation (*Axelrod Albers* and *Clemente* 1959) and in addition deaminated (*Goldstein Friedhoff* and *Simmons* 1959) The activity of monoamine oxidase (monoamine O oxidoreductase (deaminating)) is high in the corpus striatum (*Holtz* and *Westermann* 1956 *Weiner* 1960)

Dopamin is excreted in the urine in abnormally large amounts in Wilson s disease and dystonia In Parkinson s disease the dopamin excretion is abnormally small in Huntington s chorea the excretion is normal (*Barbeau* 1960 1961 *Barbeau Murphy* and *Sourkes* 1961 *Barbeau* and *Sourkes* 1961)

3 *Noradrenaline and adrenaline*

occur in the caudate nucleus in low concentrations in the dog there is about six times as much noradrenaline as adrenaline The caudate nucleus in dogs contains 0 06 μg/g noradrenaline (*Vogt* 1954) 6 per cent of the concentration in hypothalamus (*Adam* 1961)

4 *γ aminobutyric acid*

The concentration of GABA in caudate tissue from rats is 31 mg/100 g tissue Higher concentrations occur in the colliculi diencephalon and olfactory bulbs lower concentrations in cortex cerebri and medulla oblongata (*Baxter* and *Roberts* 1960) Glutamate decarboxlyase (L-glutamate 1-carboxy lyase) forms GABA from glutamic acid and this enzyme has a higher activity in the globus pallidus than in other parts of the brain (*Muller* and *Langemann* 1962) The concentration of GABA in the globus pallidus has not been reported GABA forms a shunt to the transformation of glutamic acid to α ketoglutaric acid in the *Krebs* cycle ((for references see *McIlwain* 1959) GABA has an inhibitory action on various tissues (for references see *Florey* 1961) but is not an inhibitory

ventral roots, the anterior horns and the motor nuclei of the cranial nerves (*Feldberg* and *Vogt* 1948, *Hebb* and *Silver* 1956) *Feldberg* and *Vogt* suggested that both afferent and efferent pathways often consisted of alternating cholinergic and non cholinergic neurons Stimulation of the anterior sigmoid gyrus and of the caudate nucleus (*Traczyk* and *Sadowski* 1962) in cats increased the content of acetylcholine in this nucleus and in fluid it was perfused with through the push pull cannulae of *Gaddum* (1961, *Mitchell* and *Szerb* 1962) This indicates that the caudate nucleus contains cholinergic neurons The concentration of acetylcholine in the basal ganglia' is higher than in other parts of the brain (*MacIntosh* *1941*)

The cholinesterase activity of the caudate nucleus is high (*Nachmansohn* *1940*)

Two acetylcholinesterases (acetylcholine acetyl hydrolase) with different *electrophoretic mobilities and one cholinesterase (acylcholine acyl hy* drolase) were found in the caudate nucleus and putamen dissected from human brains *post mortem* (*Bernsohn Barron* and *Hess* 1962) The proportion of choline acetyltransferase acetylcholine and acetylcholines terase is roughly the same in the different regions of the brain (dog *Burgen* and *Chipman* 1951)

Acetylcholinesterase in the corpus striatum of rats and rabbits is localized in the neuropile and on the surface of the cells Corpus striatum is stained less than the amygdala (*Gerebtzoff* 1959) Similarly *Shute* and *Lewis* (1961) found fewer cholinergic fibers in the corpus striatum than in limbic structures However manometric and bioassay determina tions of acetylcholinesterase *showed higher activities in the corpus stria* tum than in hippocampus and cingulate gyrus (*Burgen* and *Chipman* *1951*)

2 *Dopamin*

Corpus striatum contains more dopamin (3 4 dihydroxy phenylethylamine) than other brain regions The concentration in dog corpus striatum is 6 5 μg/g tissue, the average concentration in dog brain is 0 19 μg/g tissue (*Bertler* and *Rosengren 1959) In human post mortem* material the concentration of dopamin in putamen (8 25±0 82 μg/g tissue) was higher *than in the caudate* nucleus (5 74±0 41 μg/g tissue) The con centration in the globus pallidus was low (1 01±0 17 μg/g tissue) of the same magnitude as in the hypothalamus Cerebellum does not contain dopamin (*Sano* et al 1959) Lower concentrations but the same propor tions were reported by *Bertler* (1961)

DOPA decarboxylase (3 4-dihydroxy L-phenylalanine carboxy lyase)

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transmitter in the spinal cord (*Curtis* and *Phillis* 1958, *Curtis Phillis* and *Watkins* 1959, 1960 *Eccles* 1961)

5 *Serotonin*

The serotonin (5-hydroxy tryptamine) concentration in the caudate nucleus in cats and dogs is 0 72±0 2 μg/g Higher concentrations are found in limbic structures and hypothalamus, lower in cerebellum and cerebral cortex (*Bogdanski Weissbach* and *Udenfriend* 1957) The concentrations of serotonin found in dogs by *Paasonen McLean* and *Giarman* (1957) were lower than those referred to above, but the proportions were the same In the human brain *Bertler* (1961) found 0 27 μg/g in the caudate nucleus, 0 23 μg/g in putamen and 0 08 μg/g in globus pallidus

6 *Histamine*

The histamine content of the caudate nucleus in dogs is 100–180 ng/g only 13 per cent of the concentration in hypothalamus (*Adam* 1961)

7 *Piperidine*

In rat brain the concentration of piperidine is 0 05 μg/g (*Honegger* and *Honegger* 1960) The regional distribution is unknown Tritium labelled piperidine injected intravenously in rabbits is found 30 minutes later with the highest concentration in the caudate nucleus The neocortex has the second highest uptake and the lowest uptake is in thalamus and white matter Piperidine has been tested in psychiatric disorders because it antagonizes the psychotomimetic action of piperidine glycolates (*Abood Rinaldi* and *Eagleton* 1961)

B METABOLISM

The metabolic rate of the corpus striatum is high The caudate nucleus of adult dogs consumes 0 135 ml O /100 g tissue/hour (*Himwich* and *Fazekas* 1941) and the corpus striatum of rabbits 0 123 ml O /100 g tissue/hour (*Leeman* and *Pichler* 1941) this is more than other regions of the brain consume Similarly the capacity for anaerobic metabolism indicated by the rate of glycolysis is higher in the caudate nucleus and cortex cerebri than in other brain regions (*Chesler* and *Himwich* 1944) Autoradiography of the brains of alert cats, after inhalation of a radioactive, inert gas showed that the perfusion rate of the caudate nucleus

(1 10±0 08 ml/g/min) was exceeded only by cortical and thalamic sensory receiving areas (*Sokoloff* 1961)

In agreement with this high metabolic rate and despite its high capacity for anaerobic metabolism the corpus striatum is destroyed quickly in anoxia about as quickly as the cerebral cortex (*Courville 1936 Weinberger Gibbon* and *Gibbon* 1940) After carbon monoxide poisoning degeneration is most pronounced in the cortex and globus pallidus Why the globus pallidus is more often affected than the rest of corpus striatum is unknown (*Courville* 1953 *Kornyey* 1955)

The content of *riboflavin* in the caudate nucleus from man and guinea pig (4 µg/g *Leeman* and *Pichler* 1941) and the activity of *succinate dehydrogenase* (succinate (acceptor) oxidoreductase) in caudate tissue from dogs are higher than in other parts of the brain (*Burgen* and *Chipman* 1951)

The *thiamine* content in the caudate nucleus of rats is intermediate between high values in the cerebellum and low values in thalamus (*Dreyfus* 1959)

The content and the turnover of *nucleic acid* and *free nucleotides* (*Mandel Hart* and *Borkowski* 1961) and of *glycogen* (*Chesler* and *Himwich* 1943) in corpus striatum is intermediate between the high values found in the cerebral cortex and the low values in the brain stem.

C METALS

1 Copper

The copper content of corpus striatum is of interest because a disturbance in copper metabolism is believed to be present in Wilson s disease (*Cartwright* et al 1954 *Walshe* and *Cumings* 1961) The normal corpus striatum contains more copper than the cerebral cortex less than substantia nigra and locus coeruleus (*Tingey* 1937 *Cumings* 1948 *Warren Earl* and *Thompson* 1960) The different workers found different concentrations in the corpus striatum from normal human brain *Cumings* (1948) reported the highest figures similar to those found in guinea pig by *Holbrook* (1961) caudate nucleus 5±1 7 µg/100 mg dried tissue putamen and globus pallidus 8±2 25 µg/100 mg All copper in the brain is bound to protein This protein can be separated into three fractions (*Porter* and *Folch* 1957)

Copper-containing enzymes transform DOPA to melanin and the highest concentrations of copper occur in the pigmented regions nucleus niger and locus coeruleus In Wilson s disease the copper content of all

transmitter in the spinal cord (*Curtis* and *Phillis* 1958, *Curtis, Phillis* and *Watkins* 1959, 1960, *Eccles* 1961)

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SUMMARY

This chapter is an enumeration of substances occurring in the corpus striatum The caudate nucleus and putamen have higher activities of enzymes which form and destroy acetylcholine and a higher concentration of dopamin than other parts of the brain The caudate nucleus contains less serotonin than the limbic structures and hypothalamus and less γ aminobutyric acid than the diencephalon and the colliculi The caudate nucleus contains less noradrenaline adrenaline and histamine than other regions of the brain The functions that the above substances may have in the corpus striatum are unknown

The corpus striatum has a high rate of metabolism of the same order as the cerebral cortex The activity of succinate dehydrogenase and the concentration of riboflavin is higher in corpus striatum than in other regions of the brain Nucleic acid and free nucleotides and glycogen occur in higher concentrations in the cerebral cortex The concentration of thiamine in the corpus striatum is lower than in the cerebellum

Corpus striatum contains more iron and mangenese than other brain regions but only moderate amounts of copper Wilson s disease is associated with abnormal deposits of copper in all tissues the deposit in the putamen is higher than in other regions of the brain

Future studies may show whether the enzyme activities and the contents of the different substances are the same in 1) nuclei of the same histological structure as the caudate nucleus and putamen (p 31) 2) Regions with different structure but with the same or closely related function as the caudate nucleus and the frontal neocortex in primates (p 61)

tissues is increased with an especially marked increase in the putamen (*Cumings* 1948)

Copper intoxication in cats does not produce a syndrome resembling Wilson s disease Copper injected intraventricularly was not selectively taken up in any brain region, neurons were destroyed and the cats became quadriplegic and died (*Vogel* and *Evans* 1961)

In guinea pigs on a copper rich diet increased serum copper concentrations were accompanied by increased concentrations in the globus pallidus and putamen only up to a certain level The largest uptake was in the caudate nucleus and cerebellar cortex Hypercupremia, induced by tranquilizers of the phenothiazine group, produced a greater uptake of copper than the same hypercupremia induced by a copper rich diet Changes in the behavior of the animals were not reported (*Holbrook* 1961)

2 *Iron*

The globus pallidus and nucleus niger have the highest iron content in the brain (*Spatz* 1922) Corpus striatum in human *post mortem* material contains 55 mg iron in 100 g dried tissue, 5 mg/100 g is bound in hematin (10 mg/100 g fresh tissue, with a water content of 80 per cent is not bound in hematin) Globus pallidus contains 85 mg non hematin iron in 100 g dried tissue (22 mg/100 g fresh tissue water content 74 per cent *Tingey* 1937) Only one to two per cent of the iron in the brain is bound to the cytochromes (*McIlwain* 1959 p 101)

3 *Manganese*

Corpus striatum contains more manganese (0 53 μg/g fresh tissue) than any other part of the brain and has more than double the concentration of the cerebral cortex Manganese is a cofactor in certain metabolic reactions (for references see *McIlwain* 1959) Whether this can account for the distribution of manganese is not known

Manganese poisoning has been claimed to produce extrapyramidal symptoms The symptoms are stiff muscles spastic gait intention tremor involuntary laughter and a mask-like face without sensory or mental changes apart from dullness of mind The syndrome has been claimed to simulate each of the following states Parkinson s disease Wilson s disease, multiple sclerosis epidemic encephalitis mesencephalitis and carbon monoxide poisoning In animals treated with manganese lesions can be found in all parts of the brain (for references see *von Oettingen* 1935) In a human lesions were most pronounced in the brain stem and the liver (*Kober* and *Hanson* 1918)

Table 2

Autopsies after pallido-ansotomy in 23 patients

Number of lesions	Structures damaged in addition to the globus pallidus and ansa lenticularis	Rigidity and tremor diminished	Authors	Microscopic examination performed
1	None	Yes	Spiegel & Wycis 1958	+
1	None	Yes	Hausepian & Pool 1958	?
1	None	No	White McCarthy & Balin 1960	+
1	None	Yes	Smith 1962	0
1	Putamen	Yes	Smith 1962	0
1	Amygdala	Yes	Spiegel & Wycis 1958	0
1	Internal capsule	Yes	Bertrand Martinez, Poirier & Gauthier 1958	?
1	Internal capsule	Yes	Spiegel & Wycis 1958	0
1	Internal capsule	Yes	Guiot et al. 1959	?
1	Internal capsule	No	Smith 1962	0
2	Internal capsule	Yes	Nørholm & Tygstrup 1962	+
2	Internal capsule	No	Nørholm & Tygstrup 1962	+
1	Internal capsule & thalamus	Yes	Nørholm & Tygstrup 1962	+
2	Internal capsule & putamen	No	Nørholm & Tygstrup 1962	+
1	Internal capsule putamen & thalamus	No	Smith 1962	0
1	Internal capsule & nucleus subthalamicus	No [1]	Bertrand 1958	+
1	Internal capsule thalamus & nucleus subthalamicus	No	Nørholm & Tygstrup 1962	+
4	Internal capsule thalamus zona incerta & nucleus subthalamicus	Yes	Smith 1962	0
1	Internal capsule thalamus, zona incerta & nucleus subthalamicus	No	Smith 1962	0

[1] produced bilateral choreo-athetosis.

Chapter 8

PALLIDO-ANSOTOMY IN HUMANS

Lesions in the region of the globus pallidus alleviate rigidity and tremor in 60 to 80 per cent of patients with Parkinson s disease (*Guiot* and *Brion* 1952, *Narabayashi* and *Okuma* 1953, *Spiegel* and *Wycis* 1954, *Guiot* et al 1959 *Taarnhøj*, *Arnois* and *Donahue* 1960, *Bertrand* and *Martinez* 1961, *Broager* and *Nørholm* 1961, *Cooper* 1961 *Levin* et al 1961) Autopsy studies of these lesions may shed light on the pathophysiology of rigidity and tremor Not all neurons in the globus pallidus are degenerated in Parkinsonism (*Davison* 1942), and a functional deficit may result from pallidotomy Whether the surgical elimination of the remaining neurons is responsible for the subtle mental changes observed after operations for Parkinson s disease is unknown (*Riklan* and *Diller* 1958 *Riklan* et al 1960 a b, *Riklan* 1961, *Fortin* *Vanier* and *Boucher* 1961)

A SITE OF LESIONS ALLEVIATING TREMOR AND RIGIDITY

Operations aimed at the globus pallidus and ansa lenticularis have been in use for only ten years and the death rate has been low

Autopsy studies of 23 brains with unilateral and two brains with bilateral lesions in the globus pallidus have been published and correlated with the effect on rigidity and tremor (Table 2) In most cases neighbouring structures were damaged in addition to the globus pallidus *Nørholm* and *Tygstrup* (1962) reported seven cases *Smith* (1962) nine cases The remaining publications concern one or two cases each The study by *Smith* (1962) is preliminary and the results of the microscopic examination are not yet available

Of lesions extending into the internal capsule three caused lasting hemiplegia, one was at a coronal level as far caudal as the red nucleus (*Smith* 1962), two in brains with far advanced disease and diffuse degeneration in the corpus striatum and brain stem (*Nørholm* and *Tygstrup* 1962)

Rigidity and tremor were reduced by lesions small or large located in widely scattered sites A histologically verified lesion confined to the

changes seemed to be present but were not statistically significant in the 51 patients investigated The changes detected by the Rorschach test were described as follows (*Riklan* 1961) —there is a suggestion of a slight continuing decline in the motivational aspects of intelligence for both groups (left and right sided operations) and a significant deficit in somatic or body efficiency for the right brain group The personality pattern revealed no essential changes for the left brain group while those under going surgery for the right hemisphere tended to increase in emotional ex pansion but decrease in total productivity and in accuracy of perceptual structuring as compared with their preoperative functioning Quantita tive methods used for assessment of mental changes in animal studies may be adaptable to humans

A detailed study of the mental effects of bilateral lesions is not yet available

SUMMARY

A histologically verified lesion confined to the globus pallidus alleviated the rigidity and tremor of Parkinson s disease in one case and did not alleviate the symptoms in one Lesions situated mainly in the internal capsule diminished rigidity and tremor without causing lasting hemiplegia It has not been established whether the globus pallidus and its efferent connections or the corticospinal tract must be damaged to diminish rigidity and tremor Subtle personality changes were reported after unilat eral operations for Parkinsonism but apparently the changes were difficult to quantify Investigation of the mental effects of bilateral lesions are not yet available

globus pallidus in one case alleviated (*Spiegel* and *Wycis* 1958) and in a second case did not alleviate rigidity and tremor (*White, McCarthy* and *Balin* 1960) Hence it has not been established whether the globus pallidus and its efferent fibers (*Meyers* 1942), or the corticospinal tract, (*Bucy* 1958, 1961), or both must be destroyed to diminish rigidity and tremor That lesions in the internal capsule reduce rigidity and tremor without producing paralysis was anticipated by *Browder* (1948), *Browder, Kapland* and *Rabiner* (1953), *Guiot* et al (1959), *Mullan* (1960) and *Taarnhøj Arnois* and *Donahue (1960)*

B DEEP STIMULATION

Electrical stimulation through the electrode which is used subsequently to make the lesion helps to ensure reproducible results (*Walker* 1957 *Guiot* et al 1959 *Hassler* et al 1960 *Alberts* et al 1961 *Guiot* 1961 *Hausepian* and *Purpura* 1963) It may be difficult to determine *where in the brain the tip of the electrode is situated* The internal capsule cannot be used as a landmark Stimulation of many of its tracts cannot be expected to produce movements (cortical afferents interconnections between the cerebral cortex and the cerebellum and brain stem) and the topography of the tracts that produce movements is unknown The corticospinal tract is generally believed to be the most important motor tract However section of the medullary pyramids does not paralyse cats dogs monkeys and chimpanzees (p 16) Where in the internal capsule the corticospinal tract is situated is unsettled (*Hirayama* et al 1962)

The use of threshold stimuli (*Guiot* et al 1959 *Guiot* 1961 *Alberts* et al 1961) does not prevent spread of current it does not ensure that the electrode is at the site of the excited structure The threshold might be lower in a unexplored neighbouring position

C. MENTAL CHANGES

In *animals* lesions in the corpus striatum produced changes in memory and emotion (p 63 66)

In most *patients* attention wanes when the lesion is placed (*Bertrand* 1958) According to *Fortin Vanier* and *Boucher* (1961) somnolence frequently occurs after the operation, presumably not due to medication *Cooper* (1961) reported somnolence mental confusion and dulling of the intellect lasting for a week in only 8 per cent of the patients The patient s families report that sleep occurs more easily and more frequently than before *the operation* (*Fortin* et al 1961) The immediate postoperative changes are most pronounced after bilateral lesions Psychological testing about 22 days after unilateral lesions showed a general decline in intellectual functioning 10 months later preoperative performance was regained (Wechsler Bellevue Bender Gestalt, Barbeau Pinard, *Fortin* et al 1961, *Riklan* et al 1960 a b *Riklan* 1961) Personality

pathophysiology of involuntary movements and rigidity and to speculations about the normal physiology of the striatal or extrapyramidal system The corpus striatum was believed to inhibit brain regions directly concerned with movement abnormal involuntary movements were attributed to release from striatal inhibition Autopsy findings were interpreted to indicate that the corpus striatum was a center of automatic and instinctive movements associated movements etc (*Jacob* 1923)

In the nineteen forties concepts of the function of the corpus striatum were again based on animal experiments Inhibition of movements was elicited by stimulation through electrodes placed in the corpus striatum (*Mettler* et al 1939) and strychnine neuronography led *Dusser de Barenne* and *McCulloch* (1938) to suggest a suppressor circuit area 4s→nucleus caudatus→globus pallidus→thalamus→area 4 These findings were consistent with clinicopathological conclusions and were the basis of new ideas about the mechanism of tremor and choreo-athetosis (*Bucy* 1942)

PRESENT CONCEPT

The idea of the suppressor circuit has been abandoned the suppression described by *Dusser de Barenne* and *McCulloch* resembled the "spreading depression of *Leao* (1944) Inhibition of movement is due to spread of current to the internal capsule in anesthetized (*Peacock* 1954) as well as in alert cats (*Laursen* 1962 b)

There is no anatomical evidence to indicate direct projections from the caudate nucleus to the cerebral cortex (*Voneida* 1960 *Szabo* 1962) The results of electrical stimulation and recording have been interpreted to indicate direct and indirect connections between the caudate nucleus and cerebral cortex (for references see chapter 4) Precautions to detect spread of current to the structures surrounding the caudate nucleus indicate however that cortical responses to stimulation of the caudate nucleus are absent Previous findings may be attributed to spread of current to either the corpus callosum internal capsule or thalamus Similarly electrocortical arousal is absent after stimulation confined to the caudate nucleus corpus striatum is not a part of the reticular activating system (*Laursen* 1961 a b)

On the other hand there is anatomical evidence that the cerebral cortex projects to the corpus striatum and gross and single unit responses in the caudate nucleus are evoked by stimulation of the cortex (*Albe Fessard* et al 1960 a b *Laursen* 1961 a)

Sensory stimuli of different modalities evoke gross and single unit responses in the corpus striatum (for references see chapter 5) Stimulation

SUMMARY AND CONCLUSIONS

PREVIOUS CONCEPTS

Systematic investigation of the corpus striatum began with *Magendie* (1839) Neither he nor *Ferrier* (1876) distinguished between the grey substance of the caudate nucleus, putamen and globus pallidus and the white substance of the internal capsule A contralateral paresis was produced when these structures were destroyed, stimulation elicited flexion movements of contralateral extremities Bilateral ablation of the corpus striatum produced running forwards, running backwards occurred after ablation of the cerebellum

The next period (1880–1910) was characterized by attempts to sort out the functions of the corpus striatum from those of the internal capsule The main results were that movements could not be elicited by electrical stimulation of the caudate nucleus when the internal capsule had de generated and that motor defects were absent after lesions confined to the caudate nucleus Nor could effects on the autonomic nervous system which had been attributed to the corpus striatum be confirmed Solely globus pallidus was believed to have motor function, since electrical stim ulation of it evoked convulsions even when the internal capsule had degenerated (*Bechterew* 1909) Ideas about the function of the corpus striatum changed completely in the clinicopathological epoch initiated by *Cecile Vogt* (1911) and *Wilson* (1912) They described the syndrome of the corpus striatum characterized by involuntary movements and dis turbances in muscle tone without defects in sensation and intelligence Since the internal capsule (pyramidal tract) was intact in patients with the syndrome of the corpus striatum *Wilson* stated that It is unfor tunate that there is no expression in common use to indicate extrapyra midal motor disease However, matters were more complicated than originally assumed because regions outside the corpus striatum were da maged as well in cases resembling *Vogt*s and *Wilson*s syndrome in other respects These different regions together with the corpus striatum were thought to be an interconnected, integrated motor system called the striatal system by *Vogt* and *Vogt* (1919 1920) and the extrapyramidal system by *Jacob* (1923)

The clinicopathological observations led to attempts to deduce the

SAMMENDRAG

TIDLIGERE OPFATTELSER

De første systematiske undersøgelser af corpus striatums funktion blev foretaget af *Magendie* (1839) Hverken han eller *Ferrier* (1876) skelnede mellem den grå substans i nucleus caudatus putamen og globus pallidus og den hvide substans i capsula interna Ødelæggelse af disse strukturer lammede benene pa den modsatte side af kroppen elektrisk stimulation af de samme strukturer fremkaldte bøjningsbevægelser af benene på modsat side Når corpus striatum på begge sider var fjernet løb dyret fremad løb baglæns kunne fremkaldes ved ødelæggelse af cerebellum

I den næste periode (1880–1910) forsøgte man at skelne mellem corpus striatums og capsula internas funktioner Det viste sig at bevægelser ikke kunne fremkaldes ved elektrisk stimulation af nucleus caudatus når capsula interna var degenereret samt at læsioner begrænsede til corpus striatum ikke fremkaldte ændringer i dyrenes bevægelser og adfærd Heller ikke virkninger pa det autonome nervesystem som man tidligere havde tilskrevet corpus striatum kunne bekræftes Kun globus pallidus blev af *Bechterew* (1909) anset for at have en motorisk funktion idet stimulation af denne kerne fremkaldte kramper selv når capsula interna var degenereret

Forestillingerne om corpus striatums funktion ændrede sig ganske i den klinisk pathologiske epoke som blev indledt af *Cecile Vogt* (1911) og *Wilson* (1912) med beskrivelsen af corpus striatum syndromet. Dette syndrom var karakteriseret ved abnorme involuntære bevægelser og tonus forstyrrelser uden forstyrrelser af sensibilitet og intelligens Corpus striatum var degenereret og capsula interna (tractus pyramidalis) var intakt hos patienter med dette syndrom og *Wilson* erklærede "It is unfortunate that there is no expression in common use to indicate extrapyramidal motor disease Sagen var imidlertid mere indviklet end man havde tænkt sig til at begynde med det viste sig at også områder udenfor corpus striatum var beskadigede i tilfælde som klinisk lignede det syndrom *Vogt* og *Wilson* havde beskrevet Disse forskellige områder blev sammen med corpus striatum opfattet som et system af indbyrdes forbundne strukturer med en fælles funktion *Vogt* og *Vogt* (1919 1920) indførte navnet det striære" system *Jacob* (1923) navnet det extrapyramidale system

of the caudate nucleus evokes turning of the head to the contralateral side and running in circles as if the cat were searching for something Stereotyped flexion movements of contralateral extremities, elicited through electrodes in the caudate nucleus, can be attributed to excitation of the internal capsule (*Laursen* 1962 a) In unanesthetized cats spinal root to root reflexes and spindle afferent discharges are enhanced by stimulation of the caudate nucleus and globus pallidus Moderate doses of Nembutal® make the effect of stimulation inhibitory

Lesions in the caudate nucleus reduce a monkey s ability to remember whether or not food is placed in a covered cup (*Battig Rosvold* and *Mishkin* 1960) Lesions in the globus pallidus produce rapid extinction of conditioned avoidance responses in cats at a time after the operation when retention of the response has returned to normal this indicates diminished fear (*Laursen* 1963)

The function of the corpus striatum is on a high level of integration

modaliteter (referencer findes i kapitel 5) Stimulation af nucleus caudatus fremkalder hoveddrejning og løb i cirkler til modsat side som om katten ledte efter noget. Stereotype bøjningsbevægelser i modsidige ben kan også fremkaldes med en elektrode i nucleus caudatus men det skyldes stimulation of capsula interna (*Laursen* 1962 a) Hos ubedøvede katte faciliteres spinal reflexer og muskeltene ved stimulation af nucleus caudatus og globus pallidus Moderate doser Nembutal® gør virkningen af stimulationen inhibitorisk.

Læsioner i nucleus caudatus nedsætter en abes evne til at huske om mad er eller ikke er blevet anbragt i en tildækket kop (*Battig Rosvold* and *Mishkin* 1960) Efter læsioner i globus pallidus kan et be tinget undvigelsessvar afbetinges hurtigere end hos normale katte Dette forekommer på et tidspunkt efter operationen hvor hukommelsen for undvigelsessvaret er normal Den hurtige afbetingning skyldes formentlig nedsat frygt hos katte med læsioner i globus pallidus (*Laursen* 1963)

Corpus striatums funktion er således på et højt integrationsniveau

De klinisk-pathologiske iagttagelser førte til forsøg på at udlede rigiditetens og de abnorme, involuntære bevægelsers pathofysiologi Man antog at corpus striatum hæmmede de områder i hjernen som direkte havde at gøre med bevægelser, unormale, ufrivillige bevægelser kunne så forklares ved frigørelse fra ' striær hæmning Desuden spekulerede man over det striære ' eller extrapyramidale' systems normale funktion, de klinisk pathologiske iagttagelser viste, mente man at corpus striatum var *centrum for automatiske og instinktive* bevægelser, medbevægelser etc (*Jacob* 1923)

I fyrrerne blev dyreforsøg igen grundlaget for opfattelsen af corpus striatums funktion Hæmning af bevægelser kunne udløses ved stimulation med elektroder placeret i corpus striatum (*Mettler* et al 1939) og på grundlag af forsøg med strychnin neuronografi foreslog *Dusser de Barenne* og *McCulloch* (1938) en suppressor kreds area 4s→nucleus caudatus→globus pallidus→thalamus→area 4 Disse fund stemte med klinisk pathologiske konklusioner og førte til nye forestillinger om tremorens og choreo-athetosens pathofysiologi (*Bucy 1942*)

DEN NUVÆRENDE OPFATTELSE

Suppressor kreds begrebet er forladt fordi ' suppression i virkeligheden er transcortical spreading depression ' (*Leão* 1944) Motorisk hæmning, udløst fra corpus striatum skyldes spredning af strøm til capsula interna i bedøvede (*Peacock* 1954) savel som i vagne dyr (*Laursen* 1962b)

Anatomiske undersøgelser viser at nucleus caudatus *ikke sender direkte* forbindelser til cortex cerebri (*Voneida* 1960 *Szabo* 1962) Forsøg med *elektrisk stimulation og registrering* er blevet tydet som tegn på direkte og indirekte forbindelser mellem nucleus caudatus og cortex cerebri (referencer findes i kapitel 4) Forholdsregler som kan afsløre spredning af strøm til strukturer omkring nucleus caudatus har imidlertid vist at der ikke findes svar i cortex pa stimulation af nucleus caudatus Resultater af tidligere undersøgelser kan tilskrives spredning af strøm *til corpus callosum*, capsula interna eller thalamus Tilsvarende kan aktivering af elektrocorticogrammet ikke *fremkaldes af stimulation* begrænset til nucleus caudatus, corpus striatum er ikke en del af det reticulære aktiveringssystem (*Laursen* 1961 a, b)

Forbindelser fra cortex cerebri til corpus striatum er påvist i mange anatomiske undersøgelser I overensstemmelse med dette svarer neuroner i nucleus caudatus på elektrisk stimulation af cortex cerebri (*Albe Fessard* et al 1960 b *Laursen* 1961 a)

Neuroner i corpus striatum svarer på sensorisk stimulation af forskellige

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EXPERIMENTAL STUDIES ON PRESSURE AND CONTRACTILITY IN THE URETER

BY

LARS BÄCKLUND

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Introduction

Historical review

The study of the physiology of the ureter has a long history but it was not until the 19th century that the investigations took a more definite form. At first very simple methods were used but keen observations were nevertheless often made. In 1869 ENGELMANN's first extensive reports on studies of the physiology of the rabbit ureter were published. ENGELMANN's study was limited to visual observations of the ureter, but in spite of this he was able to draw conclusions, some of which are still valid today.

The further development was partly due to improved technique. Intraluminal pressure measurement came into use at the beginning of this century, and a pioneer in this field was LUCAS (1905, 1906) who used an open water manometer. TRATTNER's hydrophoragraph (1924, 1932), which was used in a more or less modified form by several other workers, represented another milestone. One of these workers was LAPIDES, who in 1948 carried out investigations on the intact human ureter. In more recent years new potentialities for measurement have been introduced by advances in the field of electronics. It will suffice to mention here the great improvements resulting from the use of pressure transducers of different types. Further reference may be made to a review by FRY (1960), in which he discussed modern physiological recording instruments with particular regard to pressure measurement.

Electroureterography may be mentioned as one of the other methods of studying the physiology of the ureter. The first to record action currents from the ureteric musculature were ORBELI and VON BRÜCKE (1910), who used external electrodes. Remarkably enough there was for a long time little interest in electro

Other essential problems in the field of ureteric physiology have concerned the origin of the peristaltic contraction wave in the upper urinary tract and the conditions under which peristalsis is maintained Some authors in particular NARATH (1951) have concluded that the contraction wave starts in the calyces of the kidney and passes downwards as a coordinated contraction wave along the pelvis and ureter Others have doubted this view especially in consideration of the fact that the calyces and pelvis or the pelvis and ureter appear to contract independently of one another The existence of a regular pacemaker for ureteric peristalsis has also been claimed by several authors

The pressure levels within which ureteric peristalsis can occur have been considered to be fairly limited and the increase in pressure necessary for the initiation of a ureteric contraction has been assessed at varying levels (see further chapter II) Most of the authors who have studied the effects of high intraureteric pressures seem to be of the opinion that when there is a sufficient *degree of distension the contractile ability of the ureter ceases* The pressure level required to produce such critical distension has been reported differently in different experiments RISHOLM *et al* (1959) expressed very interesting views on the relationship between high pressure and ureteric dilatation They stated that the decrease in amplitude in the contraction induced pressure waves with increase in the resting pressure was due to pressure damping in the fluid filled renal pelvis It has been one of the aims of the present investigation to consider further this hypothesis Intraureteric pressure ureteric diameter and electromyograms have been recorded in the dog and problems concerning the relationship between intraureteric pressure and contractility under different conditions have been studied Perfused ureters *in situ* and also ureters with intact pelviureteric communications have been studied Investigations have also been made with divided and re anastomosed ureters *in situ* with the purpose of studying the conduction mechanism in the smooth muscle of the ureter

myographs of the ureter *in situ*, and it was not until the 1950's that this method became more generally adopted, intraluminal electrodes mainly being used. Classical studies on isolated segments had already been made earlier by BOZLER (1942).

Considerable interest has long been paid to the anatomy of the ureter, and knowledge of the morphological conditions has to a certain extent influenced the interpretation of certain physiological events, especially as regards the conduction mechanism. SATANI (1919 c) made a classical study of the microscopic anatomy. During recent years electron microscopy has been used mainly perhaps in the search for an anatomical basis of a postulated conduction mechanism from cell to cell. As yet, however, certain problems, especially with regard to the innervation of the ureter, have not been completely solved. A review of the anatomical, physiological and pharmacodynamic factors which affect the activity of the ureter was made by FINKLE and SMITH (1955). Reference should also be made to a forthcoming review by KIIL (1963).

Modern views regarding ureteric function

Certain details concerning the function of the ureter were clarified at an early date. The occurrence of peristalsis has long been known and similarly that peristalsis may be stimulated by urinary flow or other mechanical factors such as pinching and stretching. The possibility of stimulating the ureter electrically was also discovered early. On the other hand there has long been some doubt as to the way in which peristalsis is regulated and how the fluid transport is mediated. While the majority of workers have considered justifiably that the ureteric contents are driven forward by a propagating contraction wave, some have held the view that the ureter functions as a suction pump in which active dilatations produce the necessary under-pressure. The role of innervation in ureteric peristalsis seems to have been overestimated earlier, and in a series of studies with isolated segments BOZLER, PROSSER, BURNSTOCK and others have shown that the conduction mechanism, at least, is independent of the nerve supply. The role played normally by the nervous components of the ureter must still be considered, however, as somewhat unclear.

Other essential problems in the field of ureteric physiology have concerned the origin of the peristaltic contraction wave in the upper urinary tract and the conditions under which peristalsis is maintained. Some authors, in particular NARATH (1951) have concluded that the contraction wave starts in the calyces of the kidney and passes downwards as a coordinated contraction wave along the pelvis and ureter. Others have doubted this view especially in consideration of the fact that the calyces and pelvis or the pelvis and ureter appear to contract independently of one another. The existence of a regular pacemaker for ureteric peristalsis has also been claimed by several authors.

The pressure levels within which ureteric peristalsis can occur have been considered to be fairly limited and the increase in pressure necessary for the initiation of a ureteric contraction has been assessed at varying levels (see further chapter II). Most of the authors who have studied the effects of high intraureteric pressures seem to be of the opinion that when there is a sufficient degree of distension the contractile ability of the ureter ceases. The pressure level required to produce such critical distension has been reported differently in different experiments. RISHOLM *et al.* (1959) expressed very interesting views on the relationship between high pressure and ureteric dilatation. They stated that the decrease in amplitude in the contraction induced pressure waves with increase in the resting pressure was due to pressure damping in the fluid filled renal pelvis. It has been one of the aims of the present investigation to consider further this hypothesis. Intraureteric pressure, ureteric diameter and electromyograms have been recorded in the dog and problems concerning the relationship between intraureteric pressure and contractility under different conditions have been studied. Perfused ureters *in situ* and also ureters with intact pelviureteric communications have been studied. Investigations have also been made with divided and re-anastomosed ureters *in situ* with the purpose of studying the conduction mechanism in the smooth muscle of the ureter.

CHAPTER II

Perfusion of the ureter in situ

There have been relatively few modern studies concerning muscular contractility during perfusion of the ureter. Isolated ureters or ureteric segments have been generally used. MURNAGHAN (1957) studied the dynamics in isolated ureters from human beings and pigs at low and moderate perfusion pressures. GOULD *et al.* (1955 *a*) used isolated ureters from water buffaloes and ATWELL and BORGSTEDT (1960) investigated the peristaltic reflex in isolated dog ureters. SATANI (1919 *b*) and SLEATOR and BUTCHER (1955) perfused the dog ureter *in situ*. The latter simultaneously recorded action potentials with intraluminal electrodes. No investigations appear to have been made on the maximal height to which the intraureteric pressure can be forced while yet retaining contractile activity in the ureter. There appears to be some divergence of opinion as regards both the magnitude of the resting pressure in the renal pelvis and the ureter and the pressure level required to induce peristalsis. Pressure levels between 0–2 cm and 12–15 cm of water respectively have been claimed (DURAND and DESCOTES 1952, DAVIS 1954, MURNAGHAN 1957, KIIL 1957, FINK and MURPHY 1957, WEINBERG and SILBERS 1958, ATWELL and BORGSTEDT 1960, WEINBERG and MALETTA 1961). In those cases where both man and dog have been investigated with the same methods fairly similar conditions have been found in these two species. No studies appear to have been made, on the other hand, as to whether the rate at which the peristalsis inducing pressure level is reached is of any importance in hastening or delaying the commencement of peristalsis. Since, however, an ability of the ureteric tissue to exhibit adaptation with a slowly rising intraluminal pressure cannot be excluded *a priori*, one aim of the present investigation was to determine whether there is any difference in the induction mechanism for peristalsis when the perfusion pressure is increased at two different rates. Intact com

munication between the renal pelvis and the rest of the ureter probably plays an important part in normal peristaltic function but for reasons given below it has been considered of value to test the activity of the ureter when isolated from direct connection with the renal pelvis The investigation has also aimed at studying the reactions of the ureter when the pressure rise is continued above the level at which peristalsis is induced and the extent to which the contractile responses are altered with increasing pressure The generally accepted view that the ureteric contractions cease on high intraureteric pressure appears to be a consequence of the partial unsuitability of the older methods With these older methods it was not possible to identify the ureteric contractions in conditions of ureteric dilatation and open communication with the renal pelvis This motivated perfusion studies with as little pressure-damping effect as possible in the perfusion system

Material and methods

Fifteen adult mongrel dogs of both sexes and weighing between 10 5 and 28 kg were used in the experiments The anaesthetic used was sodium pentobarbitone (veterinary Nembutal® Abbott) 30 mg/kg body weight being injected intravenously followed by tracheal intubation For the administration of diuretics and in case further anaesthetic was required to maintain the required depth of anaesthesia a catheter was inserted routinely into a femoral vein The abdomen was opened with a mid line incision and both ureters were cannulated distally near to the bladder with polythene catheters with an outer diameter of either 1 5 or 1 (mm and a lumen of either 0 9 or 1 1 mm depending on the ureteric dimension After cannulation the abdomen was closed In the majority of the experiments the arterial pressure was recorded with a pressure transducer connected to a catheter in the left femoral artery and the respiration was recorded intermittently by connecting the tracheal tube to a Krogh spirometer filled with oxygen The movements of the oxygen clock were transformed by means of a rotatory potentiometer to electrical potential differences The ureteric pressure was recorded through the catheters by pressure transducers the outflow from the ureter

being obstructed The transducer used was an Elema Type No EMT 472 a (Elema Schonander, Solna Sweden), which has a volume displacement of 0 3 mm^3/100 mm Hg and is linear in the region 0–300 mm Hg

As a rule only one ureter was studied in each experiment the other serving as a control for the extent of the diuresis In the first part of the experiment the pressure and peristalsis were studied in ureters where communication with the kidney was intact Sodium chloride solution 0 9 % was given intravenously as a diuretic in doses of 10–40 ml, sometimes repeated The abdomen was then reopened and the "experimental" ureter divided close to the renal pelvis A perfusion catheter was inserted into the distal side of the cut ureter (i e the proximal end of the now isolated ureteric segment), care being taken to minimize damage to the vascular supply of the ureter In order to protect the catheter as far as possible from movement artifacts it was passed through a slit in the flank In its course through the belly wall the catheter ran loosely through a short rigid plastic tube of greater diameter The mid line incision in the abdomen was then again closed The perfusion catheter was connected via a long non elastic tube to a glass burette placed horizontally which at the commencement of the experiment was adjusted exactly to the level of the ureter The burette could be raised by an elevator at two different rates i e 13 2 mm/min or 82 mm/min This made it possible to increase the perfusion pressure at rates of 0 97 mm Hg/min and 6 05 mm Hg/min respectively Sodium chloride solution 0 9 % at room temperature (22–24 C) served as the perfusion fluid During the passage through the perfusion catheter however the solution became warmed, since the abdomen was closed and the rate of flow was low The flow from the burette was closed off intermittently by means of a tap This tap was situated in the burette and was closed by turning 45 No volume displacement in the perfusion system occurred on closing the tap

In the majority of experiments the perfusion pressure was recorded with a transducer which was connected to the perfusion catheter by a three way tap In the remaining cases the hydrostatic pressure was determined by measuring the difference in level between the burette and the ureter An optical oscillograph was used for recording with mirror galvanometers (Rubstrat)

and two cameras one with a slow film speed for continuous and one with a high film speed for intermittent rapid recording In the experiments where the arterial blood pressure was not measured an ink writer (Varian G 10) was used for the ureteric pressure recordings together with a D C amplifier (pH meter PHM 22 Radiometer Copenhagen with specially modified input and output connections)

Results

With intact pelviureteric connections

Eighteen experiments were performed in all In two cases satisfactory ureteric activity was not obtained In the other sixteen cases the experiments were performed with the technique described above When a ureter with intact renal communication was occluded it was a constant finding that when the basal pressure rose the amplitude of the pressure waves increased first followed later by an increase in the frequency With a *progressively* increasing basal pressure the amplitude of the pressure waves decreased and finally became so low that the contractile activity in the ureteric muscles could no longer be identified as peristaltic pressure waves *With a sufficiently high pressure only pulse synchronous pressure oscillations were observed in the ureter* This condition usually occurred at an intraureteric pressure level of 25–30 mm Hg In exceptional cases large pressure wave amplitudes due to contractions were found even with higher pressures This appeared to be more common in young dogs and puppies A typical course in the ureteric pressure curve after occlusion may be seen in Fig 1 After intravenous infusion of sodium chloride the ureteric pressure rose regularly but no other important change was noted in the pressure curve

Perfusion at low pressures (below 15–20 mm Hg)

In the perfusion experiments the pressure increase was not started until any residual fluid had been emptied out of the ureter In half of all the experiments contractions which may have been provoked by irritation caused by the indwelling catheters were observed *In two cases* these contractions could not be stopped even after a long waiting period There appeared

being obstructed The transducer used was an Elema Type No EMT 472 a (Elema Schonander, Solna, Sweden), which has a volume displacement of 0 3 mm^3/100 mm Hg and is linear in the region 0–300 mm Hg

As a rule only one ureter was studied in each experiment, the other serving as a control for the extent of the diuresis In the first part of the experiment the pressure and peristalsis were studied in ureters where communication with the kidney was intact Sodium chloride solution 0 9% was given intravenously as a diuretic in doses of 10–40 ml sometimes repeated The abdomen was then reopened and the "experimental" ureter divided close to the renal pelvis A perfusion catheter was inserted into the distal side of the cut ureter (i e the proximal end of the now isolated ureteric segment), care being taken to minimize damage to the vascular supply of the ureter In order to protect the catheter as far as possible from movement artifacts it was passed through a slit in the flank In its course through the belly wall the catheter ran loosely through a short rigid plastic tube of greater diameter The mid line incision in the abdomen was then again closed The perfusion catheter was connected via a long non elastic tube to a glass burette placed horizontally which at the commencement of the experiment was adjusted exactly to the level of the ureter The burette could be raised by an elevator at two different rates i e 13 2 mm/min or 82 mm/min This made it possible to increase the perfusion pressure at rates of 0 97 mm Hg/min and 6 05 mm Hg/min respectively Sodium chloride solution 0 9% at room temperature (22–24 C) served as the perfusion fluid During the passage through the perfusion catheter however the solution became warmed since the abdomen was closed and the rate of flow was low The flow from the burette was closed off intermittently by means of a tap This tap was situated in the burette and was closed by turning 45 No volume displacement in the perfusion system occurred on closing the tap

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Left ureter

mm Hg

100
90
80
70
60
50
40
30
20
10
0

Pressure

NaCl

1 min

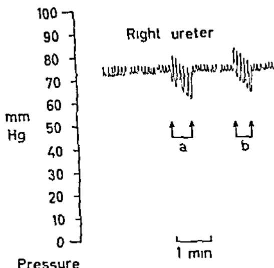
NaCl

…g. 1 *Ureteral pressure variations after total occlusion of the distal end of the ureter* (Pelviureteric connections intact.) The basal … sure, which is represented by the minimum value of each pressure wave, rises rapidly. The character of the curve changes at a … ssure of about 30 mm Hg when the amplitude of the pressure wave induced by the contractions becomes too small to be measured … e ureteric pressure subsequently varies synchronously with the respiratory movements and the arterial pressure, which are not … orded in the picture but were observed visually. The increase in pressure resulting from intravenous injections of 0.9 … NaCl solu… … can also be seen. Recorded with an ink writer.

to be no correlation in this respect between the size of the ureter and that of the catheter inserted Large ureters *often reacted to* a catheter which in other experiments had been inserted into a smaller ureter without causing irritation With the slow pressure increase (0 97 mm Hg/min) it was found that in all cases except three, which will be described separately below, *ureteric peristalsis commenced at pressures between 1–2 and 16 mm Hg* As soon as peristalsis had definitely started and with regular frequency, the experiment was discontinued and the perfusion pressure reduced again to zero, after which the ureter was allowed to empty itself freely Only small quantities of fluid (0 4–1 ml) ran from the burette into the ureter during the experiments When the ureteric activity appeared to have ceased once more a new perfusion was commenced with a rapid increase of pressure (6 05 mm Hg/min) There again the peristalsis started at approximately the same pressure level as with the slow pressure increase In several cases there was no difference and on a few occasions the peristalsis was produced at lower pressures during the slow increase in pressure The three cases that had reacted differently with the slow increase also deviated from the typical response to a rapid increase of pressure On the whole they reacted more slowly, with a rapid rise in pressure the contractions did not start until the pressures reached 20 25 and 50 mm Hg respectively, and with the slow rise 40, 30 and 70 mm Hg respectively The ureter that did not start contracting until the pressure had reached the considerably raised level of 50 mm Hg was found on examination at the end of the experiment to have thickening of the muscle layer and pronounced trabeculation and a thickened mucosa in the bladder In the subsequent part of the experiments however none of these three ureters differed from the rest of the ureters included in the material

Perfusion at high pressures

On continued increase of pressure above the level at which the ureteric contractions had begun *two principal activity patterns were observed* Either the contractions occurred regularly with frequencies of about 5–10/min or 6–8 contractions were observed to alternate with inactive phases of varying length The latter was most commonly seen Regardless of the pattern exhibited by



F g 3 The sam experiment as in Fig 2 but with a higher pressure and later There has been a sta i of long duration with a perfus on pressure maintained at o r 40 mm Hg for 60 minutes There were regular contractions the press ire wa e a npl tudes of wl ich ro.e markedly during the two pe iods when the per fus on burette was closed (a b) When the burette was closed tl e basal pressure fell d ie to leakage from the catl ter

wave amplitude increased when the tap was closed Fig 3 illus trates the persistence of contractility even on prolonged in traureteric hypertonia Fig 4 shows a perfusion experiment with a rapid pressure increase The amplitudes of the pressure waves are seen to decrease on rising pressure and are hardly distinguish able at 95 mm Hg before the burette is clo ed off (Fig 4 *b*)

During the periods when the respiration frequency was re corded *no relationship between respiratory movements and ureteric peristalsis could be shown* The frequencies sometimes coincided without this changing the peristaltic pattern or short ening the phases of inactivity

With the system used perfusion pressures higher than 150 mm Hg could not be applied and any remaining pressure variations due to contraction could not be distinguished with certainty from artifacts due to re piratory movement or other external disturb ances exerting pressure against the ureter or the indwelling catheter In tests to find out how high a pressure dog ureters can

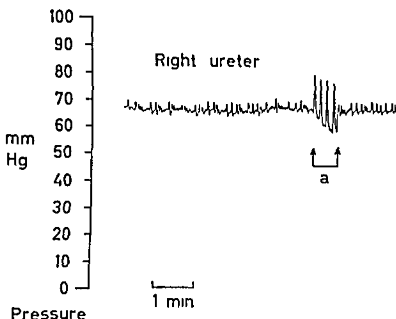


Fig 2 Pressure recording from an experiment in which the ureter was perfused in situ There are small somewhat irregular pressure variations their amplitude was increased markedly during the time the burette used for perfusion was closed (a) *Recorded with an ink writer*

the individual ureter, both the frequency and the amplitude of the pressure waves decreased with increasing perfusion pressure When the pressure was again reduced the contraction frequency and the amplitude increased again In several cases however both the amplitude and the frequency were lower while the pressure was being reduced than at the corresponding level during pressure increase The maximal perfusion pressure that could be applied without loss of the contractile power varied between 50 and 125 mm Hg In five cases the pressure was 100 mm Hg or more The ureters were thus capable of functioning for a fairly long period with high intraluminal pressures In the majority of the experiments the perfusion pressures were maintained for an hour or more

At different pressure levels the burette tap was closed and the ureteric pressure wave could not then be transmitted to the free fluid surface of the burette *It was found constantly that the pressure wave amplitudes then increased markedly* Fig 2 shows the ureteric pressure curve before and after closing of the tap *The contraction frequency was unchanged but the pressure*

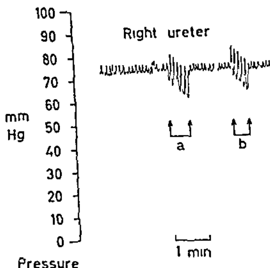
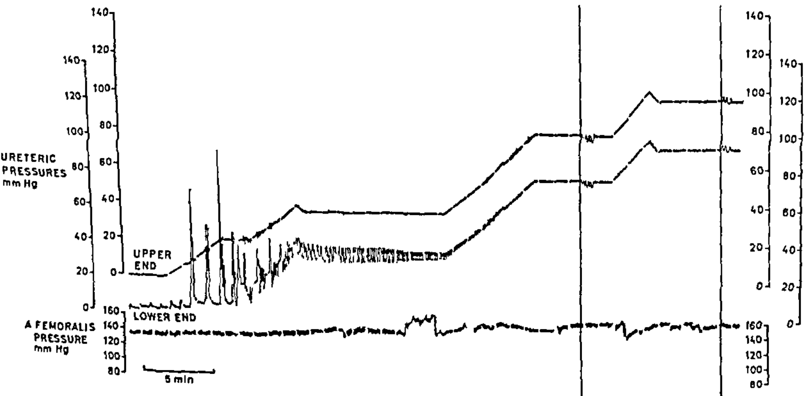


Fig 3 The same experim t as in F g ? b t with a higher pressu e and later Ther has been a sta of long duration with a perfu ion pres ure maintained at over 40 mm Hg for 60 minutes There were regular contraction the pressure wave ampl tudes of wh ch rose markedly during the two p riods wl en the per fus on burette was clos d (*a b*) When the burette was closed the basal pressure fell due to l akag fr m the cath ter

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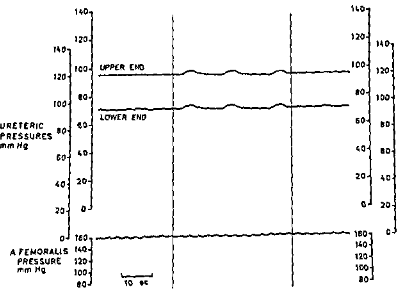
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URETERIC PRESSURES mm Hg
A FEMORALIS PRESSURE mm Hg
UPPER END
LOWER END
5 min

a

Fig 4 1 f ion rj r me t rec r led w th a t ally gr j1 n t howing th j re ur in th perf t n th t r (jj r n l) a i l t tally r th ureter (low n l) Fig 4 rec r h g th a l fil s j d Fig 4b n o l ig with a l igl r f lm p l at th t r f th me i l s et al l s e i i Fig 4a In th low re ording it ca i t e n l w j r tal i t er ase uecessiv ly in frequ ncy wl le the a i plitu le lecrea s with ri ng pressure At bou t 60 mm Hg the a npl it ide is very mall The j erfu cn l ur tte was cl sed at l oth the s rt all i s in Fig 4a r ulting in a t npo ary increase in the j ssu ave a j l tu le whi l decl ned again sl r the l ur tte was reoj ened (t marked) The lifference i tl e j r ures ave a pl tu le is better s n n th r j l re o l g s he i the l urette was lo e l be tw n the t s e ti il l i

Note the l ul l er h ate cale The pres ure in the upj an l lo s r r l s o the same The p ure i j el from th f r ral art ry were lampe l giving a m ean value



b

tolerate without rupture, a fluid pressure corresponding to 370 mm Hg was applied in two cases without any leakage or macroscopic damage being caused Not until the pressure was increased to a level well beyond the response range of the transducer did the ureteric walls rupture

Discussion

Peristaltic patterns

The peristaltic pattern of the ureter after acute total occlusion and with intact pelviureteric communication involves firstly increasing basal pressure, followed by increase in the amplitude and the frequency of the pressure waves The latter increase especially at pressures of about 10–15 mm Hg Similar observations have been made by several other authors Initial increases in frequency and amplitude are described by GOULD *et al* (1955 *b*), among others It has been discussed whether increased ureteric flow results in an increase in tone alone (MORALES *et al* 1952) or also in an increase of frequency (HANLEY 1953) With further rise in pressure the amplitude of the pressure waves decreases progressively (TRATTNER 1932, LAPIDES 1948, KIIL 1957, RISHOLM *et al* 1959, BOYARSKY and MARTINEZ 1962 and others) The interpretation offered by most authors for this finding is that the activity of the ureteric musculature gradually ceases with increasing distension, and that under normal conditions the urine would flow out of such a ureter by virtue of the pelvic pressure MORALES *et al* (1952), who studied the reactions of the ureter at different flow rates stated that with a high flow the ureter is so dilated that no contractions are possible With sufficiently high intraluminal pressure the whole ureter becomes dilated and free fluid communication is established with the renal pelvis BOYARSKY and MARTINEZ (1962) have demonstrated this by means of urography It has been often found that when there was an open fluid communication in the upper urinary tract ureteric pressure oscillations were completely synchronous with the arterial pulse and there were no visible pressure peaks due to contraction This phenomenon has been described previously by several authors and will be discussed in more detail in chapter III This free fluid

communication occurs fairly constantly at 25–30 mm Hg in dogs This agrees with the conclusion of HENDERSON (1905) that in dogs the ureteric contractions disappear at pressures corresponding to 26–32 mm Hg

A few workers however, have also described ureteric con tractility at higher intraluminal pressure levels TRATTNER (1932), in experiments with his hydrophoragraph found that contrac tions died out at a level of 38–70 cm water GOULD *et al* (1955 *b*) noted ureteric contractions at 50 cm water when the pressure was forced to increase by manual compression of the urinary bladder SLEATOR and BUTCHER (1955) recorded action potentials from ureters during perfusion with pressures up to 25–30 cm water The peristaltic waves were then at a maximum and tended to come in groups DAVIS (1957) stated that the peristaltic waves disappeared completely at pressures above 54 mm Hg Reports on the contraction ability in isolated perfused ureters at still higher pressures have been published by LUCAS (1908), who observed that a segment of a dog ureter was capable of raising a column of Ringer s solution 92 cm and MURNAGHAN (1958) who demon strated the activity in a megaureter at pressures of up to 80 cm water

The damping effect of the renal pelvis

RISHOLM *et al* (1959) observed strong ureteric activity at pressures of about 40–45 mm Hg when the open communication between the renal pelvis and the ureter was interrupted with a ligature They considered that the ligation simply eliminated a damping effect of the pelvis The procedure of closing off com munication with the pelvis by a ligature involved however some displacement of fluid into the ureter It is conceivable that this might involve a trigger effect by causing extension of the ureteric walls It is thus not precluded that a temporarily extingui hed peristalsis can be re started in this way Closing off with the burette tap does not change the fluid content in the ureter and is therefore a better method After closing the ureter appeared to retain its tension since the pressure recorded in the perfusion system remained unaltered or decreased only very insignificantly

The damping theory put forward by RISHOLM seems to agree well with the present results If the natural renal pelvis is eli

minated and perfusion is carried out from an artificial "renal pelvis" with rigid walls in the form of a glass burette, it should be expected that pressure variations due to contractions would be found at pressure levels where no pressure waves can normally be identified in the ureter The results found in the experiments are in agreement with this supposition It is true that the amplitudes decrease progressively, but the damping had not been completely eliminated in the system In closing off the burette with the tap, however damping should be decreased further even if it does not disappear entirely, and the results obtained with an increased pressure wave amplitude at the moment when the burette was closed, clearly confirm this assumption *It is thus clear that on perfusion of an isolated ureter pressure waves due to contraction can still be found at 100–125 mm Hg If this ureter were in free fluid communication with the renal pelvis, however, it would exhibit the classical reaction of decreasing pressure wave amplitudes and, at 25–30 mm Hg, "cessation of peristalsis"* The muscularly weak renal pelvis does not appear to be able to resist large pressure increases and a damping effect due to regurgitation of fluid is therefore obtained as soon as an open communication has been established

A certain degree of damping however must also take place in the ureter Since the whole organ does not contract simultaneously a rapid pressure wave distribution takes place in the fluid filled dilated ureter, and the temporarily inactive parts become able to distend further A perfusion technique such as *that used here with pressure measurement alone* is therefore not perfect for determining the level to which the pressure can be raised with retention of contraction ability The boundary line as regards the power of the ureteric muscles to contract against progressively increasing pressure has not yet been determined It is clear however that it exceeds that pressure that may possibly occur in the ureter in physiological or pathological conditions Further, there must be a considerable *margin between the pressure* which can be overcome by the muscles and that which the muscular and elastic elements in the ureteric wall resist in a purely passive way before rupture and macroscopic breaks occur In two control experiments a pressure of 370 mm Hg was exerted without any rupture of the ureter Corresponding experiments

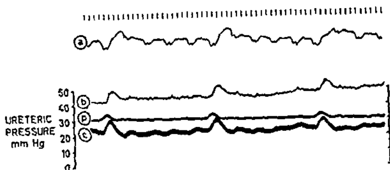


Fig. 5. Retrograde peristalsis during ureteric perfusion. Simultaneous recording of the ureteric diameter at three different points (*a*, *b* and *c*). Point (*a*) was situated nearest to the kidney and (*c*) nearest to the bladder. The large upward deviations indicate contractions. (*p*) is the pressure in the ureter distal to (*c*). The pressure increases begin before the contractions at (*c*), which in their turn appear before the contractions at (*b*) etc. There are small respiration induced variations in ureteric diameter. The time intervals in seconds are marked in the uppermost part of the figure. Recording with a Visicorder.

on human ureters (BOONE and SMITH 1955) showed pressure tolerances up to 200 mm Hg without any change in the elastic properties of the ureteric wall.

Peristalsis at high pressures

The question of whether the contractions shown by the ureter at high pressures constitute a peristaltic wave running the length of the whole organ, or an antiperistaltic wave, or whether these are merely local strong contractions, cannot be answered using the method described. At low pressures there is probably a normally propagated peristaltic wave, since fluid movement was constantly observed in the glass burette a few seconds before the pressure increase was recorded in the distal part of the ureter. It is probable that the pelviureteric communication is of great importance for the initiation of normal peristaltic waves. The existence of a pacemaker for the peristalsis has been suggested by many authors and will be discussed in detail in Chapter III. In perfusion experiments on the rabbit (BÄCKLUND, unpublished data) it was found difficult to produce peristalsis when the communication between the pelvis and ureter was broken. In dogs it is clearly possible to initiate a contraction wave merely by a

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pared with the rapid increase It is possible that a difference would be observed if a pressure increase were induced at a rate of for example 0 1 mm Hg/min and that the muscle cells would then have time to become ' adapted The effect in this case would be that contraction would appear first after a greater amount of distension than when a rapid but small pressure increase was produced This will be discussed further in chapter VI

proximal pressure increase At high pressures there is a dilated ureter with free fluid communication between the two pressure transducers and the pressure wave is therefore recorded at practically the same time in both places In *some experiments* there was, however, a tendency towards an earlier rise in pressure distally, which *either indicated antiperistalsis or that only the* distal part was contracting Many authors have also maintained *that the distal third of the ureter is last in ceasing to contract* on rising pressure (LALIDES 1948, MURNAGHAN 1957) Fig 5 *shows* a perfusion experiment with a modified technique in which the ureteric diameter was recorded in three different places For the method reference may be made to Chapter III The contractions appear to travel in a *retrograde* direction Observations of peristalsis after division of the ureter were made by SHIRATORI and KINOSHITA (1961 *b*) They found that when the ureter was divided at the pelviureteric junction retrograde peristaltic waves running through the whole ureter occurred

The relation between perfusion pressure and contractile response

The magnitude of the perfusion pressure at the commencement of peristalsis in the individual ureters varied In the two cases where there was spontaneous peristalsis, it is possible that the catheters caused *irritation that stimulated* contractions The effect of an indwelling catheter on ureteric peristalsis has often been discussed, *and it has been questioned especially* whether free flow and normal peristalsis can remain undisturbed under these *conditions (see KIIL 1957 for further references)* TRATTNER (1932) described spasms after the insertion of catheters The *spasms ceased within a few minutes* however either spontaneously or on intraureteric injections of sodium chloride It seems reasonable *to believe that irritation from catheters* was of secondary significance in these perfusion experiments In the great majority of cases an *intraluminal rise in pressure* and ureteric distension are required to produce peristalsis There are individual variations in the magnitude *of the contractile response* and this probably partly explains the relatively wide distribution obtained in this investigation and also *the divergent* results obtained in previous studies Neither does extension sensitivity appear to be noteworthily *different in the slow* pressure increase used com

minimize damage to the vascular supply to the ureter Polythene tubes with terminal holes and with an external diameter of either 1 5 mm or 1 6 mm and an inner diameter of 0 9 mm or 1 1 mm respectively were used as catheters The catheters were inserted about 1 cm proximally and were secured with a ligature After cannulation the ureters were allowed to drain for a few minutes for estimation of the diuresis on each side In the case of very slow ureteric flow rates 0 9 % sodium chloride solution was administered intravenously in doses of 10–40 ml to increase diuresis When a satisfactory flow of urine was obtained one of the ureters was connected to a pressure transducer (Elema EMT 472 a see page 10) and the ureteric pressure was recorded during a few minutes occlusion to assess the ureteric activity The stasis was then released and an electrode inserted into the wall of the ureter 0 5–2 5 cm above the tip of the ureteric catheter In a number of experiments a piece of silver wire about 0 1 mm thick isolated but for the tip surface in a glass capillary the outer diameter of which varied between 0 2 and 0 35 mm was used as an electrode A small piece of fine mesh earthed iron net placed under the ureter served as a reference electrode In the remainder of the experiments a concentric needle electrode (13 K 51 Disa Elektronik A/S Herlev Denmark) was used The Disa electrode was 30 mm long with an outer steel sheath of 0 45 mm diameter and an inner isolated platinum wire the active area of which was approximately 0 04 mm The steel sheath served as the reference electrode and was earthed In all of the experiments a special earthed electrode was also inserted into the left hind leg of the animal

Eighteen experiments were performed altogether and in thirteen of these the changes in the ureteric diameter were recorded with an indicator constructed specially for the purpose (Fig 6 a) The claws of the indicator were placed around the ureter at the level where the electrode had been inserted (Fig 6 b) In two experiments a further indicator was also placed 25–30 mm proximally to the electrode Both the electrode and the diameter indicators were held in position by being clamped to rigid bars and in order that the abdominal viscera should not displace the measuring instruments the viscera were packed in muslin compresses wet with sodium chloride and held aside by hooks

CHAPTER III

The contractility at high intraureteric pressures with intact pelviureteric connections

As already mentioned in the previous chapter, the decrease in amplitude of the ureteric pressure waves associated with increase in intraluminal pressure has been interpreted by the majority of workers as due to a weakening and finally an inhibition of peristalsis Since, however, the perfusion experiments showed that muscular contractility can occur even at very high intra ureteric pressures a series of experiments was performed the purpose of which was to study the relationship between pressure and ureteric muscular activity when the pelviureteric connec tions were intact, and where more criteria for ureteric contrac tions than pressure waves alone were required

Material and methods

Altogether sixteen adult dogs of both sexes and weighing be tween 8 5 and 25 kg were used for the experiments They were anaesthetized with intravenous injections of veterinary Nembutal® (Abbott) in a dosage of 30 mg/kg body weight The left femoral vein was cannulated to facilitate intravenous infusions A catheter was inserted into the right femoral artery for recording the ar terial pressure with a pressure transducer or a mercury mano meter Either an oxygen filled Krogh spirometer adapted as described in chapter II or a balloon placed under the diaphragm and connected to a pressure transducer for marking abdominal pressure variations were used for pneumotachography

The abdomen was opened with a long mid line incision from the ensiform process to the symphysis pubis After puncturing and emptying the urinary bladder both ureters were cannulated as near to the bladder as possible great care being taken to

munication between the renal pelvis and the ureteric catheter When necessary the diuresis was stimulated with sodium chloride infusions In seven of the experiments succinylcholine iodide (Celocurin® iodide Vitrum) in a dose of 1 mg/kg body weight was administered intravenously to produce total blockade of the striated muscle

The recording apparatus varied somewhat The electromyogram was recorded via a cathode follower either on a two channel oscilloscope with or without simultaneous recording of the diameter variations and photographed with a Du Mont Polaroid Land camera type 2620 or together with all the rest of the traces on a multichannel recorder The input time constant of the cathode follower was 4 μsec and the amplification factor 0 83 The active electrode was connected by a double shielded cable to the grid of the cathode follower tube and the inner cable shield to its cathode The outer shield was connected to the reference electrode and earthed By using a shield connected to the cathode the capacitative effects in the electrode cable are kept as low as possible thus improving the signal reproduction

In most of the experiments an optic oscillograph (described in chapter II) was used as a writer in the other experiments a fourteen channel oscillograph of the Honeywell Visicorder type 906 A-1 was used with D C amplifiers (Grass Low Level Pre amplifiers) for the ureteric pressure transducer and diameter indicators

Results

The time course of the ureteric peristalsis after occlusion showed the same pattern as in the experiments described in chapter II After a period of increasing frequency and amplitude the amplitude of the pressure waves again decreased with increasing pressure As a rule when there was free fluid communication between the renal pelvis and the ureter only pressure oscillations synchronous with the arterial pulse and other arterial pressure variations were obtained

An action potential occurred constantly before each pressure wave and as an expression of ureteric contraction a decrease in the diameter of the ureter was obtained after each action potential The close relationships between action potentials ureteric

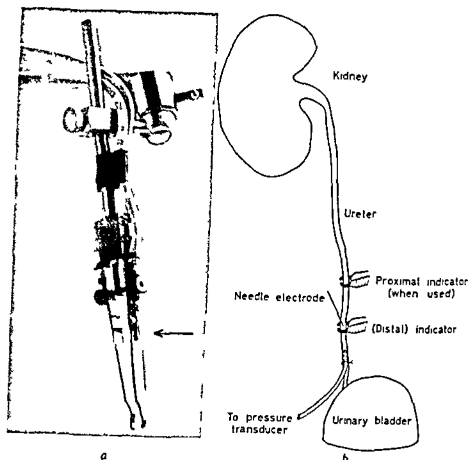


Fig 6 Indicator for measuring variations in ureteric diameter (a) It consists of two tongues of phosphor bronze 10 cm long and 0 6 cm broad They are attached at one end to a wedge shaped brass block Their free ends are narrower and shaped so that together they resemble a claw At the arrow the tongues are ground thin so that they are very flexible Strain gauges coupled as part of a bridge are mounted on the thin regions of both tongues The tongues have some resilience but very little force is required to move them and all bending occurs at the thin regions The movements are thus transduced via the strain gauges into electrical information With the aid of two screws placed just above the thin regions the claws can be adjusted so that they just enclose and very lightly touch the ureter The instrument thus subsequently follows radial variations in the ureter The schematic diagram (*b*) shows where these indicators were placed

When all preparations were complete the ureter was totally occluded by connecting the catheter to the pressure transducer and the ureteric pressure, action potentials (AP) and diameter variations were studied at the same time as also were the arterial pressure and respiration An attempt was made to obtain an intraureteric pressure sufficiently high to ensure free fluid com

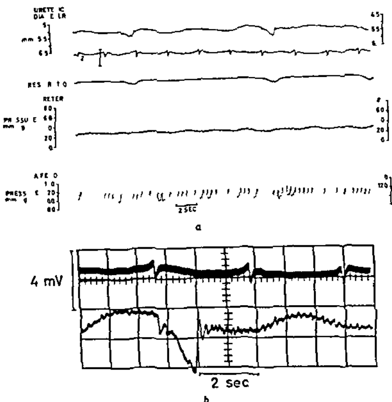


Fig 8 R gula co t tio in a ureter with i tact pelviureteric con ectio s and a pr ss re of 25–30 mm Hg There are small pressure variations synchronous with the art l pulse tog ther with a small and regular increase after each act on potent al Th d ameter recording also hows respiration dependent changes (Opt al oscill graph) Fig 8b shows athode ray osc llo cope traces of act on potential (bo) an l liam t r ariations at the time of the second respiratory mo m nt in Fig 8

contractions and pressure increases at moderate intraureteric pressures (5–20 mm Hg) were studied separately and are treated in chapter IV

With pressures of over 25–30 mm Hg the pressure wave amplitudes became so small that they could not be identified with certainty *The action potentials and ureteric contractions however continued to occur with an essentially unchanged appearance* The different parts of Fig 7 show the ureteric activity at different intraluminal pressures With low pressures (Fig 7 *a*)

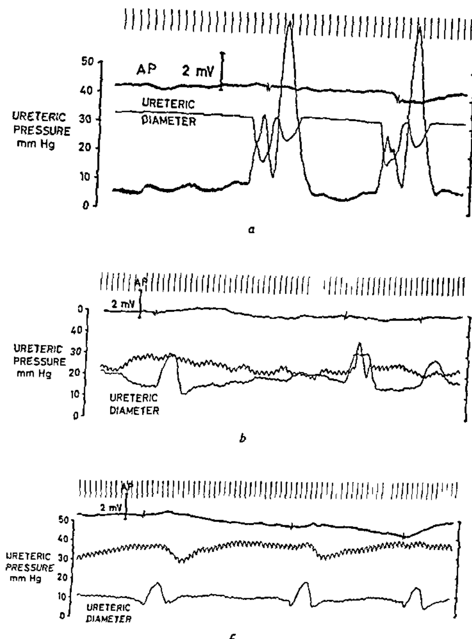


Fig 7 The relationship between the action potential and the variations in ureteric diameter and pressure (Pelviureteric connections intact) Upward deviations in the diameter recording indicate contraction and downward deviations dilatation The curve in Fig 7*a* is discussed in more detail in Chapter IV Note that in 7*b* the ureteric pressure rises with the second contraction (incomplete damping) *In both 7b and 7c* the ureteric *pressure varies synchronously with the* arterial pulse and respiration The latter were studied visually and are not recorded here The large periodic decreases in ureteric pressure in 7*c* are due to respiration *The* uppermost *marks are time intervals in seconds Recordings with* Visicorder

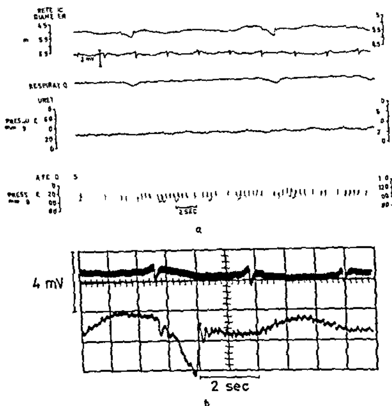


Fig 8 Regula co ilr tion in a ret r with intact pel ureteric connections and a pressu of 25 30 mm Hg Th re are small pressure variations synchronous with th arterial pulse tog ther with a small and regular increase alter each action potent al Th di n ter *recording* also stows respirat n dependent chang (Optical osc ll graph) Fig 8b shows cathode ay oscilloscope traces of act on pot nt als (bov) an l l am t r variat ons at the tum of the second respiratory movement in F g 8

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TABLE 1 *The frequency of ureteric contractions immediately after the beginning of occlusion (initial frequency) and then after two consecutive periods*

Experiment No	Initial frequency per min	Occlusion time in min	Final pressure in mm Hg	Final frequency per min	Occlusion time in min	Final pressure in mm Hg	Final frequency per min
1	*17*	34	30	*20*	70	50	*15*
2	*10*	23	30	*8*			
3	*18*	*28*	*40*	*10*	43	*50*	*9*
4	*9*	*3*	35	*20*	17	45	*20*
5	*8*	32	30	*24*	56	45	*10*
6	*12*	*50*	30	*10*	90	30	*6*
7	*30*	60	55	*20*	150	60	*6*
8	*17*	*30*	*45*	*17*	*90*	75	*13*
9	*9*	30	50	*15*	60	60	*12*
10	*8*	60	30	*13*	90	35	*13*
11	*17*	80	60	*12*	160	50	*12*
12	*24*	30	25	*24*	60	35	*24*
13	*17*	*32*	25	*15*	*50*	50	*12*
14 a	*15*	70	40	*11*	110	65	*12*
14 b	*10*	45	40	*12*	60	60	*5*
15	*6*	85	35	*5*	150	55	*4*
16	*3*	30	35	*3*	50	40	*3*
17	*7*	*35*	*50*	*7*	*50*	*50*	*6*
18	*6*	60	25	*10*	100	40	*10*

For example in experiment No 1 the frequency was 20 cycles/min after 34 minutes when the pressure was 30 mm Hg and later (70 minutes after occlusion) with a pressure of 50 mm Hg the frequency was 15 cycles/min Experiment No 14*b* was carried out on the same ureter as 14*a* and was begun 15 minutes after the conclusion of 14*a*

large pressure amplitudes during and after every action potential are seen At high pressures these pressure waves are reduced and disappear while the action potentials remain (Figs 7 *b* and *c*) at the same time contractions can be seen clearly in the diameter recording

Fig 8 shows the contractility in conditions of free communication between the pelvis and ureter, and the oscillations synchronous with the arterial pulse The diameter variations in

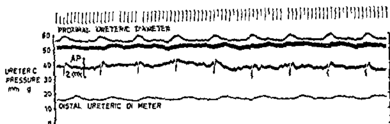


Fig 9 Forn a d movi g peristalsis with a u et ric pressu e of 50 mm Hg (Pelvi ureteri connections intact) The ureteric pressure curve shows slow waves due *to respirat* on with superimposed very small oscillat ons synchronous with the arterial pulse The electromyogram was reco ded from the *same level as was* the d stal ureteric d am ter The course of events is as follows (from left to r ght) *First a proximal* ureteric contraction is seen followed by an action po tential (distal) preceding the distal contraction *A few seconds later a second* proximal contraction takes place and the chain of events is repeated etc Upper most time marking in seconds Recorded with Visicorder

Fig 8 are rather unclear due to the low sensitivity of the re cording system used The diameter recordings were therefore *displayed* simultaneously on a cathode ray oscilloscope with high amplification and distinct contractions were observed During *the* recording of Fig 8 *b* a respiration artifact unfortunately *appeared* so that one ureteric contraction was completely obliter ated

The relationship between the action potentials and contractions may also be seen in Figs 9 and 10 *b* which *were recorded* under conditions similar to those in Fig 8

The frequency showed a tendency to *decrease* on continued increase in pressure or on prolonged stasis (at the same pressure) The difference was small however in several cases *The highest pressure reached was 75 mm Hg and ureteric activity was still retained at this level* the frequency being 13 contractions per minute and the period of total occlusion 90 minutes A summary of the data regarding frequencies ureteric pressure and occlusion periods may be seen in Table 1

The duration of the individual contractions varied between 2 and 5 seconds The amplitude of the action potential was as a rule 1–2 mV with extreme values of 0 5 and 4 mV and a duration between 0 25 and 0 60 seconds (Figs 13 *c* 14 *b* and 16 *c* chapter V) The latent period between the beginning of the action poten tial and that of the ureteric *contraction* (measured as incipient

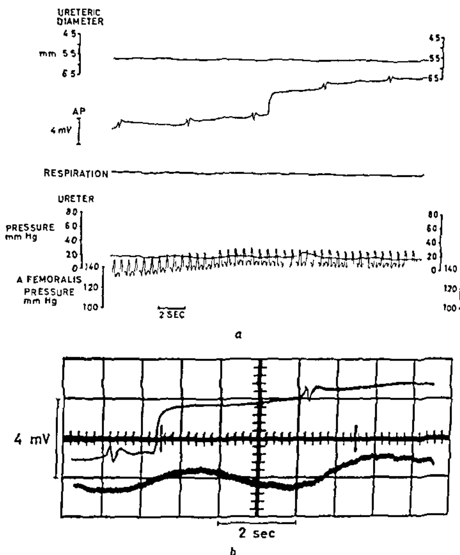


Fig 10 Ureteric activity four minutes after Celocurin administration (Pelviureteric connections intact) There are regular ureteric contractions but no pressure increase (*Fig 10a Optical oscillograph*) *In Fig 10b the third and fourth con*tractions with their preceding action potentials (above) are illustrated with the cathode ray oscilloscope traces in order to demonstrate more clearly the variations in ureteric diameter (lower curve)

diameter reduction) was 0 4–0 5 seconds The latent period showed a tendency to increase in prolonged experiments where the action potential also tended to decrease in amplitude and change in form

In several cases there was no difficulty in observing visually the downwardly propagated peristaltic waves in spite of high

intraureteric pressures There was good correlation between the passage of a visibly propagated peristaltic wave past the electrode site and the appearance of an action potential In the two cases where two diameter indicators were used a contraction was constantly recorded at the proximal indicator before the action potential and contraction at the distal indicator (Fig 9) A similar sequence of the contractions was observed in a few experiments of similar type in which three diameter indicators and no action potential recording were used

In all cases where Celocurin was used the dog was allowed to breathe pure oxygen for 20–30 minutes before the Celocurin was administered and in this way no respiratory assistance was required during the few minutes immediately following the commencement of paresis (apnoeic oxygenation) Recordings were made when all striated muscle activity including respiratory movement had ceased In all cases except one the ureter remained entirely unaffected and no alteration occurred in the frequency pressure or electromyogram Fig 10 shows the ureteric activity after the commencement of apnoea The diameter variation on the oscillograph recording (Fig 10 *a*) are small and appear more clearly in the oscilloscope picture (Fig 10 *b*) In one case there was a pronounced rise in arterial pressure (vasoconstriction?) following the Celocurin administration and the ureteric pressure decreased simultaneously When the ureteric pressure dropped below 25 mm Hg distinct pressure variations due to contractions occurred

No relationship between the respiratory movements or the abdominal pressure variations due to them and the ureteric activity was found apart from mechanical artifacts Nor was there any relationship between the arterial pressure variations and the ureteric activity

Discussion

Electromyography at high intraureteric pressures

Electroureterograms have been used by several previous workers as a means of studying ureteric function Intraluminal electrodes have been used most frequently (HANLEY 1953 FRANKSSON and PETERSÉN 1953 CALAFATI et al 1954 SLEATOR and

BUTCHER 1955, BUTCHER and SLEATOR 1955, BUTCHER and SLEATOR 1956, COREY *et al* 1956, BUTCHER *et al* 1957, and WEINBERG and SIEBENS 1958), while in a few investigations electrodes have been applied to the adventitia (ORBELI and v BRUCKE 1910, WEINBERG and HOFFMAN 1961, WEINBERG 1962) or inserted intramuscularly (BAKER and HUFFER 1953, GARG *et al* 1959, SHIRATORI *et al* 1959, SHIRATORI and KINOSHITA 1961 *a* and *b*, SHIRATORI and CHIBA 1961) An intraluminal electrode technique is very liable to artifacts since good contact with the ureteric epithelium should be maintained, and at the same time distension by a catheter that is too large must be avoided The different types of extra luminal electrodes give more exact information about the action potentials and are thus to be preferred It is true that an intra muscular electrode damages the ureteric tissue locally, but the recording is made the whole time from a well defined static point in contrast to juxta adventitial electrodes over which the ureter is able to slide

The present technique is very similar to that used by SHIRATORI and his collaborators, and appears to give almost identical results in dogs with respect to the form amplitude and duration of the action potentials SHIRATORI described three types of waves with amplitudes varying between 0 5 and 1 5 mV and durations of 0 2–0 4 seconds BAKER and HUFFER (1953) used another tech nique A needle (hypodermic needle No 28) was used as an active electrode and the indifferent electrode was placed in the skin of the abdomen The action potentials obtained in this way had an amplitude of about 0 2 mV thus lower than those found by SHIRATORI The duration was also shorter i e about 0 1 second The different results appear to be due to differences in the method of recording Under good conditions action potentials similar to those of Shiratori are obtained with an intraluminal technique (SLEATOR and BUTCHER 1955) and with adventitial electrodes (WEINBERG and HOFFMAN 1961)

The two most common types of action potentials in the Japanese material were also most common in the present investigation while the third form of wave (Fig 14 *b*) which has the lowest amplitude and was observed by SHIRATORI and collaborators espe cially in conditions when the peristalsis was weak occurred most often with high pressures or in prolonged occlusion experiments

The form of the action potentials thus seems to vary with the condition of the muscle cells but the recording of electromyograms does not *per se* necessarily involve serious reduction in function The most prolonged experiment in this material extended over 150 minutes while SHIRATORI stated that is was possible to obtain the spikes unchanged in appearance for three hours The position of the electrode in the ureteric wall seems to be an essential factor as regards the appearance of the action potentials Electromyograms with different types of electrodes are fairly divergent results and it is reasonable to suppose that a varying intramural electrode position might give variations in the form of the action potentials The appearance of the individual spike is therefore probably less important than the fact of its *existence* The occurrence of potential variations alone is not however necessarily evidence of ureteric contractility It only indicates that localized depolarizations (or very local non propagated contractions) occur in the smooth muscle but whether regular contractions arise can only be determined by some form of *simultaneous diameter indication If both an action potential and a subsequent decrease in diameter are recorded, this must be regarded as proof that a contraction has taken place* The experiments showed that even when the intraureteric pressure rose to a height such that the amplitude of the contraction dependent pressure variations decreased and finally ceased because of damping the contractions remained *The most usual way of identifying the ureteric activity i e the measurement of ureteric pressure changes alone must thus be regarded as inadequate at such intraureteric pressure levels when there is open communication with the renal pelvis and the damping effect of the latter influences the observed pressure changes*

Roentgen contrast investigations of the ureter (BENJAMIN *et al* 1956) also appear to be unreliable when a certain degree of distension has been exceeded This is to be expected since with high pressure and pronounced dilatation the lumen of the ureter is not completely closed during contraction (GRAVES and DAVIDOFF 1923) It is probably quite difficult to identify on a roentgenogram partial decreases in diameter on ureteric contractions On the other hand the contractions can be observed when the degree of dilatation is more moderate BENJAMIN *et al* (1956) demon

strated ureteric contractions with cinefluorography in obstructed dog ureters in the presence of moderate dilatation

The effect of intravenous NaCl infusions on the ureteric pressure

As shown in chapter II ureteric activity can be demonstrated in perfusion experiments at high hydrostatic pressure levels In experiments with intact pelviureteric communications the ureteric pressure seldom rises spontaneously above 45 mm Hg After a more or less rapid pressure increase, the pressure required to *dilate the ureter and establish free* communication *between the* ureter and pelvis is reached In addition, if the diuresis is sufficient, the pressure increases further to a level which should correspond approximately to the filtration pressure in the glomeruli The "pattern of free communication" which is shown in these cases by the ureteric pressure recording completely reflects the changes in arterial pressure With every pulse beat the pressure rises and falls to some degree, and slow arterial pressure oscillations and variations in the arterial pressure synchronous with respiration are very well reflected These phenomena have been described by several authors (RISHOLM *et al* 1959, TAYLOR *and* ULLMAN *1961,* KIIL *and* AUKLAND *1961 b,* BOYARSKY and MARTINEZ 1962 and others) Because of the stasis in the ureter the kidney is affected by the increased pelvic pressure, and the diuresis decreases (SELKURT *et al* 1952, KIIL and AUKLAND 1961 *b*) If a diuretic is given the pressure can be forced *higher* than 45–50 mm Hg even without change in the arterial blood pressure TAYLOR and ULLMAN obtained maximal pressures of 148 mm Hg after intravenous injections of 23% mannitol in normal saline If infusions of isotonic NaCl are repeated in moderate amounts the ureteric pressure increases sharply within ½–1 minute after each infusion and can be raised to 100 mm Hg without difficulty It seems to be predominantly a local intrarenal reaction that causes the high pressures after the administration of normal NaCl Infusions of hypotonic NaCl also probably have this effect KIIL and AUKLAND (1961 *a*) gave 0 45% NaCl intravenously *in quantities* of 4–8 ml/min and obtained maximal pelvic pressures of 134 cm water If the diuresis was maintained with repeated infusion of 5% glucose the pelvic pressure increased but not in sharp steps or if a fluid with hypernormal colloid

osmotic pressure but containing no electrolytes is administered (10% Dextran) a transient reduction of the pelvic pressure is obtained (BACKLUND and NORDGREN to be published) Since it is improbable that pressures above 100 mm Hg occur in the urinary tract either physiologically or under pathological conditions it has not been considered worthwhile to attempt to reach such pressure levels experimentally The study of the ureteric activity has therefore been mainly confined to the pressure region of 30–60 mm Hg

Ureteric peristalsis at high intraluminal pressures

Only the lower part of the ureter was studied In the majority of the experiments the ureteric diameter was only measured at one place and it is possible that the activity which was observed was local to the distal ureter and therefore simulated peristalsis As mentioned in chapter II it has been claimed that the power of contraction ceases last in the lower third *In several experiments however a fully visible peristaltic wave was observed and in the cases where several diameter indicators were used a distal contraction was preceded by a proximal one* (Fig 9) *Antiperistalsis* was not demonstrated with certainty *in this series of* experiments but it *probably occurred* It *has* been *observed* among others by TRATTNER *(1932)* SLEATOR and BUTCHER (1955) and MELICK *et al (1962)* The latter studied human ureters *On the other* hand SHIRATORI and KINOSHITA (1961 *a*) did not *observe any* anti peristaltic discharges in normal dog ureters

The fluid transport along an occluded *ureter* does not cease even if there is total distal occlusion The continually persistent contractions produce a *circulation of* fluid within the closed system Furthermore *return transport from the renal* pelvis to the blood can take place (RISHOLM and OBRINK *1958*) apparently predominantly via the lymphatics (FOLDI *et al* 1959)

The effects of the experimental procedure

Of *especial interest is the contraction* frequency in the occluded ureter A slowly *decreasing* frequency was found when the pressure was high and/or the occlusion was maintained for a long period This was probably a result *of* the experimental procedure At the same time a decrease in the amplitude of the potentials

strated ureteric contractions with cinefluorography in obstructed dog ureters in the presence of moderate dilatation

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observed that the ureteric muscles can hypertrophy proximally to a ureteric stone (WILDBOLZ 1952), and after partial ureteric occlusion with dilatation (BENJAMIN *et al* 1956)

Pacemakers in the ureter

The existence of a pacemaker for ureteric peristalsis has been discussed and NARATH (1951) and MORALES *et al* (1952) among others held the view that the contraction waves began in the calyces and were propagated to the ureter via the renal pelvis SLEATOR and BUTCHER (1955) also described this finding but considered it hazardous to conclude from their results that a renal pacemaker normally exists BOZLER (1942) on the other hand found in the extreme renal end of isolated ureters a small region with high spontaneous activity from which the propagated ureteric contractions arose BOZLER regarded this as a pacemaker situated at the pelviureteric junction It was not the intention in the present investigation however to determine from what part of the urinary tract the propagated ureteric contractions usually originate but to study whether the respiratory movements or changes in the arterial pressure can constitute a trigger mechanism In a few cases the frequencies of respiration and peristalsis coincided but no definite relationship was found PFEIFER (1919) with roentgen contrast investigations of the renal pelvis found good correlation between the respiratory movements and the filling variations of the renal pelvis According to PFEIFER deep inspiration could provoke a peristaltic wave If this occurs it is probably only an occasional coincidental occurrence One of the aims of the experiments with Celocurin was to eliminate any possible respiratory stimulation of the ureter in no case did total respiratory paresis result in any change in the ureteric activity Nor did Celocurin affect the electromyogram The same results were also obtained by SHIRATORI and CHIBA (1961) who after the administration of succinylcholine chloride in doses of 1 mg/kg body weight found no change in the electromyogram from dog ureters

The part played by the arterial pressure in ureteric peristalsis is indirect It may be considered to be a 'supply pressure' for diuresis and also determines the appearance of the communication pattern If the effect of the arterial pressure on the kidney

was seen in a number of cases, and also sometimes a protraction of the latent period between the action potential and the commencement of contraction. The latent period was considerably longer than that described by SLEATOR and BUTCHER (1955). Using another technique in which the electrical and mechanical activity were not measured at the same place they calculated a latent period of 0.1 second from the action potential to the beginning of mechanical activity. In the present investigation, however, the ureter was not in its natural milieu. Both cooling and drying may contribute to a tendency to a slower reaction, and such effects are additive to the effect exerted by operation trauma and increase in pressure. The anaesthetic used, on the other hand, probably exerted no untoward effect on the ureter. Care was taken to produce an adequate depth of anaesthesia. Too light an anaesthesia can lead to profuse recording artifacts, a phenomenon which has also been described by BAKER and HUFFER (1953). These authors studied the electromyogram from dog ureters under anaesthesia with ether or intravenous sodium seconal, and also the importance of anaesthetic depth. No effect on the electromyogram was shown in any of the cases studied.

The durations of the individual contractions varied, but the range was similar to that found by BOYARSKY and MARTINEZ (1962). With low diastolic pressures they recorded a systolic duration of 2–5 seconds, and in studies on man BORS and BLINN (1955) found a variation of the ureteric systole of between 1.5 and 4 seconds.

In spite of the unfavourable external conditions the ureters were able to contract for long periods at high intraluminal pressures. No definite conclusions can be drawn, however, regarding the length of time that an occluded ureter is able to maintain peristalsis, but a period of several hours seems a reasonable minimum estimate. In a series of experiments with partially obstructed and dilated dog ureters BAKER and HUFFER (1953) found only small electromyographic changes after 2–4 months. The peristalsis was reported as being forceful with a normal rate, but the intraureteric pressure was not determined. No great degree of muscular atrophy occurs in chronically obstructed ureters; there is predominantly an increase in the amount of fibrous tissue (STEIN and WEINBERG 1962). It has also been

CHAPTER IV

Ureteric diameter variations and electromyograms at low intraluminal pressure levels

A number of studies of ureteric peristalsis at low and moderate intraureteric pressures have been reported in the literature In recent years electromyography most frequently with intraluminal electrodes and with simultaneous pressure recording without occlusion of the ureter has been used to study the point of origin and the rate of propagation and also the importance of the rate of flow Both roentgenograms and intraluminal detectors sensitive to diametric changes have been used to study ureteric contractions A combination of pressure measurements diameter recordings and electromyography does not appear to have been utilized however for the analysis of ureteric function at physiological pressure levels In order therefore to study more closely the local electrical and mechanical events accompanying the passage of a peristaltic wave a series of experiments with measurement of the intraureteric pressure and simultaneous recording of electroureterograms and diameter changes has been performed

Material and methods

Nineteen adult dogs of both sexes and weighing between 8 5 and 31 kg were used for the *experiments* The same operative technique was used as described in chapter III In those cases however where several *diameter indicators* were used a larger portion of the ureter was freed in order to ensure the best conditions for the indicators placed at the selected site A ureter which has been inadequately dissected *out* cannot move freely and the diameter recording is then *made considerably* more difficult

The ureters were cannulated in the same way and with the same types of catheter as *described* in chapter III The ureteric pressure arterial pressure and respiration were also recorded with

is decreased by vasoconstrictors or by compressing the renal artery the pressure falls abruptly in the pelviureteric system and contraction dependent pressure waves are seen, probably as an expression of the fact that free fluid communication between the pelvis and ureter has been interrupted in some way (BACKLUND and NORDGREN, to be published) With the exception of these effects the arterial pressure does not appear to influence the ureter, and no relationship has been shown between the contraction frequency and the variations in arterial pressure

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the same methods as before Intravenous injections of 0 9% sodium chloride solutions were given as a diuretic when required

Altogether twenty two experiments were performed, and in fourteen a single indicator of the type described in chapter III was used for diameter recordings In two experiments two in dicators placed 25 mm and 30 mm apart, respectively, were used, and in the remaining six experiments three indicators were used, the distance between the proximal *and intermediate indicators* was 15–23 mm, and between the intermediate and the distal 16–38 mm The distance between the tip of the ureteric catheter and the distal diameter indicator varied between 5 and 25 mm The greatest distance, measured from the tip of the catheter to the most proximally placed indicator, was 75 mm

For recordings of the electromyogram an electrode was in serted into the wall of the ureter between the claws of the most distal diameter indicator In a few cases however, the electrode was placed somewhat proximally or distally to this level A silver electrode was used *in ten cases and a platinum electrode in* the remaining twelve The electrodes were constructed as described in chapter III The electromyogram was also recorded as de scribed in the previous chapter

In ten experiments the optic oscillograph described in chapter II was used for recording, and in the remainder a Visicorder 906 A 1 with D C amplifiers (Grass Low Level Preamplifiers) for the ureteric pressure and diameter detectors The experiments were performed in such a way that before the recordings from a ureter were commenced, the urine was allowed to run freely from *the ureteric catheter During this time the diuresis* was estimated roughly, and the action potentials and diameter variations were assessed The ureteric catheter was then connected to a pressure transducer (Elema EMT 472 a) no urine being allowed to escape from the ureter An attempt was made to obtain moderate pres sure levels (5–20 mm Hg) so that the relationship between the action potentials ureteric contractions and pressure rises could be studied

Results

There were certain constantly recurring difficulties Careful preparation was required to obtain good electromyographic re

cordings and at the same time satisfactory diameter measurements from two or three levels It was sometimes impossible to complete an experiment without making slight adjustments of the ureteric position in the indicator claws As in the investigations described in chapter III the placing of an electrode in the ureteric wall at the site of an indicator resulted in a local decrease of movement In control experiments when the electrode was removed the diameter variations increased In spite of the presence of the electrode however the deflections were usually sufficiently large for the diameter change to be identified with certainty

The form amplitude and duration of the action potentials were the same as described in chapter III The range of variation of these parameters as recorded was large and by changing the position of the electrode in cases where the potentials were small a considerably higher amplitude was often obtained

Pressure variations The occurrence of diphasic pressure waves with peristalsis

The pressure waves were of two principal types Either a single pressure wave occurred after each action potential or as in the vast majority of cases there was a pair of pressure waves In the latter case the first wave was as a rule of lower amplitude and shorter duration than the second wave (Fig 11) The interval between the waves varied considerably and was sometimes so great that they appeared entirely separate In other cases the interval was so small that the waves were almost confluent In some cases the first wave had a greater amplitude than the second but it was never of longer duration The action potential belonging to the wave complex always appeared during the first wave Its precise localization could vary but it was never earlier than the beginning of the first wave In some cases a triphasic pressure wave complex appeared To judge from wave form and time relationship to the action potential the first of these three waves was an extra wave while the next two corresponded to the ordinary diphasic complex Thus the action potential occurred at the same time as the second wave of the triphasic complex

These different types of peristaltic waves were not unique for this experimental series but were observed in all the present

the same methods as before Intravenous injections of 0.9% sodium chloride solutions were given as a diuretic when required

Altogether twenty two experiments were performed, and in fourteen a single indicator of the type described in chapter III was used for diameter recordings In two experiments two indicators placed 25 mm and 30 mm apart, respectively, were used, and in the remaining six experiments three indicators were used, the distance between the proximal and intermediate indicators was 15–23 mm, and between the intermediate and the distal 16–38 mm The distance between the tip of the ureteric catheter and the distal diameter indicator varied between 5 and 25 mm The greatest distance, measured from the tip of the catheter to the most proximally placed indicator was 75 mm

For recordings of the electromyogram an electrode was inserted into the wall of the ureter between the claws of the most distal diameter indicator In a few cases however, the electrode was placed somewhat proximally or distally to this level A silver electrode was used in ten cases and a platinum electrode in the remaining twelve The electrodes were constructed as described in chapter III The electromyogram was also recorded as described in the previous chapter

In ten experiments the optic oscillograph described in chapter II was used for recording, and in the remainder a Visicorder 906 A 1 with D C amplifiers (Grass Low Level Preamplifiers) for the ureteric pressure and diameter detectors The experiments were performed in such a way that before the recordings from a ureter were commenced, the urine was allowed to run freely from the ureteric catheter During this time the diuresis was estimated roughly and the action potentials and diameter variations were assessed The ureteric catheter was then connected to a pressure transducer (Elema EMT 472 a) no urine being allowed to escape from the ureter An attempt was made to obtain moderate pressure levels (5–20 mm Hg) so that the relationship between the action potentials, ureteric contractions and pressure rises could be studied

Results

There were certain constantly recurring difficulties Careful preparation was required to obtain good electromyographic re

cordings and at the same time satisfactory diameter measurements from two or three levels It was sometimes impossible to complete an experiment without making slight adjustments of the ureteric position in the indicator claws As in the investigations described in chapter III the placing of an electrode in the ureteric wall at the site of an indicator resulted in a local decrease of movement In control experiments when the electrode was removed the diameter variations increased In spite of the presence of the electrode however the deflections were usually sufficiently large for the diameter change to be identified with certainty

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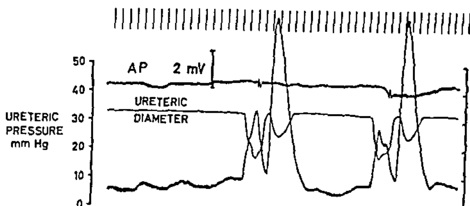


Fig 11 The diphasic pressure wave associated with peristalsis in a ureter with its connection to the pelvis intact During the first smaller pressure peak there occurs an action potential and at the same time (at the distal site of measurement) the ureteric diameter is increased (down going deflection) The second pressure peak is due to contraction at and distal to the level at which the electrode is situated In spite of the muscular contraction the ureter dilates appreciably even simultaneously with the maximum of the second pressure peak *This is a result of the obstruction in outflow* Uppermost time marking in seconds Recorded with a Visicorder (The picture is the same as Fig 7a)

ureteric studies when a recorder with a sufficiently fast paper speed was used

With increasing ureteric pressure the amplitude of the pressure waves decreased independently of whether they appeared single or in pairs As a rule the first wave of a pair disappeared before free fluid communication between the ureter and pelvis was established

Diameter variations during the passage of the peristaltic waves

*Ureteric diameter recordings showed different patterns depend*ing on the type of peristaltic wave and also on the intraureteric pressure

At pressures up to about 10 mm Hg the ureteric diameter was so little increased during inactivity compared with the collapsed resting state at zero pressure that a contraction was unable to produce any measurable diminution in diameter In the distal ureter the fluid already present inhibited compression and thus the contraction occurring was almost isometric It was therefore very usual that the ureter became somewhat dilated during a contraction at the distal measuring point At higher pressures

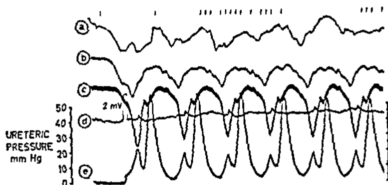


Fig 1 Regular contractions in a ureter with intact pelviuretic connections *Recording with three diameter indicators at different sites* (*a*) proximal (*b*) somewhat more distal (*c*) at the electrode level (*d*) is the electromyogram and (*e*) the intraureteric pressure Note the successive dilatation phases in *a* *b* and *c* An action potential and a small pressure rise occur simultaneously with the dilatation at (*c*) Uppermost time in seconds Recorded with a Visicorder

the ureter became more and more dilated and the contractions became more clearly manifest

In peristalsis with biphasic pressure waves the first wave coincided with a marked dilatation of the ureter during which period the action potential also occurred (Fig 11) Only exceptionally was this dilatation so small that it could not be identified with certainty Dilatations were also observed when the ureter was not occluded and there was no obstruction to emptying The second longer pressure wave occurred together with a significantly smaller dilatation (at low pressures) or with a contraction (at higher pressures)

The propagation could be studied when two or three diameter indicators were used The propagated change consisted first of a larger dilatation followed immediately by a smaller dilatation or a diminution of the ureteric diameter (Fig 12) As a rule the first pressure wave (pressure measured at the distal end of the ureter) in the diphasic curves coincided with the diminution at the upper or middle diameter indicators (see Fig 14 *a* chapter V)

When the basal pressure was increased the dilatation in parallel with the first wave disappeared while the action potential remained unaffected

In those cases where the peristalsis was accompanied by only a

single pressure wave it was nevertheless observed that a dilatation of the ureter preceded the pressure wave, the action potential occurring with the dilatation With low pressures there was also an additional but much smaller dilatation which occurred at the same time as the pressure rise

Only in exceptional cases were neither ureteric dilatation nor a pressure rise observed at the time of the action potential

Velocity of propagation and frequency of contraction

The velocity of propagation in the ureter was determined in the eight experiments where two or three diameter indicators were used In all cases the velocity was constant in a particular experiment and in different experiments varied between 12 mm and 20 mm per second The distance between which the measurements were made varied between 25 mm and 75 mm

The use of more than one diameter indicator made it possible to determine the contraction frequency in different parts of the ureter It was a common finding in at least some part of an experiment that the, or those, indicators which were placed proximal to the electrode showed a frequency double that of the action potential recording The difference could also occur between the proximal and intermediate indicators while thus the latter and the distal indicators had the same frequency The frequency change *sometimes occurred very suddenly and* was of relatively short duration In a few recordings the frequency in the distal segment was only $^1/_3$–$^1/_4$ of that in the proximal part These frequency differences were not associated with any particular pressure level

The occurrence of antiperistalsis was also studied Retrograde peristaltic waves were unusual but they did sometimes occur As a rule they were occasional waves which turned at the ureteric catheter and then went backwards

The action potential frequency was observed in some experiments to alter so that when the pressure rose to 10–20 mm Hg the spikes appeared not only before the pressure waves but also in their terminations and without a further pressure rise Another type of arrhythmia was *the appearance of spikes in pairs shortly* before a contraction This variation was unusual and occurred at higher intraureteric pressures

Discussion

Diameter variations in contraction of the ureter

A peristaltic contraction wave in the ureter as a rule means a wave of contraction of the ureteric musculature propagated from the pelvis down to the urinary bladder The circular musculature in contracting produces mainly a narrowing of the ureteric lumen while contraction in the longitudinal muscles produces a shortening of the ureter but probably not a complete occlusion of the lumen According to SATANI (1919 c) there are in the ureteric wall an outer and an inner layer of longitudinal musculature and an intermediate layer of circular muscles The circular muscles predominate in the middle third of the ureter and while diminishing in the distal third are nevertheless present right down to the entrance in the bladder wall

The anatomical distribution of the circular and longitudinal muscles in different regions of the ureter produces variations in the forces available for the compression of the lumen and thus it is probable that the ability to propagate the ureteric contents also varies from place to place With such a situation there may be a tendency for the formation of pools in certain parts of the ureter At least with low intraureteric pressures the lumen is pressed together completely with ureteric contraction and the ureteric contents are pressed as a bolus in a distal direction Since however the ureteric lumen in rest is entirely collapsed the downward displacement of a bolus must mean that the ureteric walls are forced apart and thus a local intraluminal pressure rise must occur MELICK *et al* (1962) have by cinefluorography and simultaneous pressure measurement followed the passage of boluses in human ureters They observed that the ureteric walls both above and below the bolus were completely collapsed and that a pressure rise could be recorded only where the walls were distended by the bolus The cinefluorographic technique was also used by BENJAMIN *et al* (1956) and SWENSON *et al* (1959) who also found that the ureteric lumen was completely compressed during contraction BENJAMIN *et al* stated that after contraction the lumen was opened again by the next contrast injection arriving from above The dilatation of the ureter that occurs in front of a contraction wave has been described earlier (PROTOPO-

pow 1897, Orbeli and v. Brücke 1910, Anderson 1951, and others), and has been of importance in the theory that the ureter functions as a suction pump (Lucas 1908, Sleator and Butcher 1955).

If the ureteric diameter is measured continuously the passage of the bolus should be recorded as a dilatation, and when the ureteric contraction passes the measuring point the lumen is again obliterated and the diameter regains the resting dimension, with the assumption that the entire ureteric content can be emptied distally. This is exactly *the situation which was found in the* experiments with the diameter indicators placed proximal to the electrode and at such a level that allowed a urine bolus to be pushed past in its entirety.

The conditions are different if the ureter is in stasis at a measuring point (e.g. distally near the catheter). When a contraction wave arrives, the bolus cannot be pushed on further and the ureteric lumen cannot be collapsed again completely. It must *therefore be expected that after the initial dilatation there is a* reduction in diameter, corresponding to the contraction, which cannot become total or subtotal before the muscular contraction in the ureteric segment immediately above has subsided sufficiently to allow the urine to be pressed backwards. When the ureter is slowly filled through diuresis an increasingly great part of the distal ureter becomes unable to obliterate the lumen completely. In this way free fluid communication through the *whole of this part is obtained and when a muscular contraction* occurs in the distal part the fluid column can be pushed backwards through this dilated region to a part of the ureter since no ureteric occlusion hinders its passage. Contraction of the ureteric musculature distally should then be manifested as a small but distinct diameter reduction as compared with the value between contractions. This also occurred at pressures of about 10–20 mm Hg. At the same time the initial dilatation phase became smaller *and disappeared. This also seems reasonable since* it is difficult to believe that an observable bolus can be pushed into the dilated and fluid filled ureteric tube. Thus the effect of muscular contraction in this region should be *manifest* only as a pressure rise, probably small and damped. Nevertheless it is possible that there was a small dilatation although none was

recorded This might be due to the low sensitivity of the indicators VAN CITTERS (1960) was able to show in model experiments that in a fluid filled elastic tube of the dimension of the aorta the front of a pulse wave is associated with a dilatation of the walls of the tube The ureteric contractions were however propagated and possible mechanisms to explain this will be discussed below

Pressure variations with ureteric contraction

As mentioned above on the passage of a urine bolus into an empty ureter a certain intraluminal rise in pressure should occur when the ureteric walls dilate This event corresponds to the course in the diphasic peristaltic wave pattern which constituted the most common finding in the investigation When the stream of urine is injected downwards into an empty ureteric segment the bolus jet should give a rapid moderate rise in pressure (bolus jet wave) varying with the power causing it and being succeeded by a more pronounced pressure wave when the contraction arrives at the tip of the ureteric catheter A bolus jet can probably be very forceful In experiments with exteriorized bladders MORALES *et al* (1952) observed that the urine could spurt more than 30 cm high from the ureter

The latent period between a bolus jet wave and a pressure wave (rise in pressure due to contraction at the same level) probably varies with the excitability of the smooth musculature the velocity of the bolus propagation and the ability of the ureter to dilate rapidly on intraluminal pressure increases (i e volume-pressure relationship) The fact that the mutual relationships between a bolus jet wave and a pressure wave showed such great differences can be explained by differences in anatomic structure predominance of circular or longitudinal musculature possible tissue damage or other factors In some cases there was no bolus jet wave and in these conditions the monophasic peristaltic pattern appeared Since as a rule however there was an initial dilatation the monophasic pattern appears to be only an extreme variant of the *normal diphasic pattern* Diphasic and triphasic waves were recorded together with single waves by RATTNER *et al* (1957) in man They obtained diphasic waves in all parts of the ureter Small pressure rises preceding the contractions were also

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of the diameter measurements Such a sequence was never definitely observed but cannot be excluded since electromyographic information from a proximal site is also required to establish it Spontaneously higher activity in the uppermost part of the ureter has been described however by several authors (BOZLER 1942 GOULD *et al* 1955 *b*) and could play some role at least in part in the varying frequency The ability of different ureteric segments to contract completely independently of one another, observed by TRATTNER (1932) may constitute yet another factor

The action potentials exhibited another form of arrhythmia with high pressures in certain cases they appeared either in pairs before a contraction or an extra action potential appeared at the end of a prolonged pressure wave before the basal pressure had been regained It is probable in both cases that the prevailing tension in the ureteric wall produced extra depolarizations On the whole at high intraureteric pressures the ureteric activity may be related to the continuous stimulation of the musculature provided by the internal tension Whether propagation occurs because the ureter behaves as though it were a syncytium or is mechanically induced either by means of extension in the neighbouring cell on contraction or provoked by a small local rise in pressure will not be discussed here but will be treated in more detail in chapters V and VI

The propagation velocity of the ureteric contractions

The propagation velocity in the ureteric musculature has been mainly investigated previously by electromyography In dogs SHIRATORI *et al* (1959) found a variation between *38 and 56* mm/sec while SHIRATORI and KINOSHITA (1960 *a*) reported higher velocities in the middle part of the ureter than in the remaining areas According to these authors the distal propagation velocity of the peristaltic wave was between *21 4 and 29 7* mm/sec They found velocities of 20–45 mm/sec in antiperistaltic waves which had arisen after division at the pelviureteric junction (1960 *b*) PROSSER *et al* (1955) found a velocity *of 30–40* mm/sec in dog ureters while CALAFATI *et al* (1954) noted 20–30 mm/sec Cooling and drying may be mentioned as possible explanations for the lower velocity in the present study BAKER and HUFFER (1953) and BUTCHER et al (1957) obtained a reduced velocity under

described in man by KIII (1957), who used pressure measurement catheters which did not obstruct the urinary flow

When the ureteric pressure rises the bolus jet wave would also, as is the case with the dilatation phase, be expected to disappear This was found to be so, and similarly, the pressure wave itself also disappeared gradually This phenomenon has already been discussed in chapter III and will therefore not be discussed further here

The occurrence of the action potential during the dilatation phase and bolus jet wave at lower pressures was constant *It is thus only on occasions with increased tension in the smooth muscles (as a rule due to increased intraluminal pressure) that a spike appears and a contraction is provoked* In certain experiments where the frequency was higher at a proximal than at a distal measuring point, stronger dilatations and/or greater bolus jet waves were seen when the contraction was provoked distally than in those cases in which the lowermost segment remained inactive (see Fig 13 *a*, chapter V) *This seems to indicate that the smooth muscle cell need to be stimulated with a certain minimal degree of extension if they are to respond with contraction*

Arrhythmias in the peristalsis

Different forms of arrhythmia were observed The fact that the frequency was sometimes *higher in the proximal region has* already been mentioned Another additional possible reason for *this is that when the ureteric content is pressed backwards* by a distal contraction this produces by extension of the ureteric *wall a new peristaltic wave that is able to continue forwards* until it encounters a refractory region The refractory period *for the ureteric musculature* has been stated by PROSSER *et al* (1955) to be 1–3 seconds and by ICHIKAWA and IKEDA (1960) as 1 5 *seconds* It is possible that this period may be longer in a region that has been damaged by experimental preparations and the electrode puncture Cooling of the ureter may also have similar effects Reasons such as these do not explain however why for example frequency ratios between two indicators could suddenly change to 2 1 3 1 or 4 1 If these integral frequency ratios were due to antiperistaltic waves provoked from the distal part, it would be possible to determine this by the time sequence

CHAPTER V

The peristalsis in an anastomosed ureter

It was mentioned in chapter IV that peristaltic propagation in the ureter is possibly maintained by the excitation of distal muscle cells by means of extension or intraluminal pressure increase on contraction in the more proximal muscle cells. In order to study this problem more closely and to eliminate all myogenic, nervous or humoral influences coming from above, a series of experiments were performed in which the ureter was divided and re-anastomosed via a short catheter without direct contact between the two cut surfaces.

Material and methods

Thirteen adult dogs of both sexes weighing between 10 and 33 kg were used for the experiments. They were anaesthetized with intravenous injections of veterinary Nembutal® (Abbott) in doses of 30 mg/kg body weight. In prolonged experiments the Nembutal dose was repeated to retain an unchanged depth of anaesthesia.

The same operative technique was used as in the experiments described in chapter IV. Recordings of respiratory movements and the arterial blood pressure were made as described in chapter III. For cannulating the ureters the same type of polythene catheters were used as before. The tip of the catheter was inserted 5–10 mm proximally from an incision made near to the urinary bladder. Care was taken to minimize damage to the vascular supply of the ureter.

Altogether sixteen experiments were performed. In ten cases the ureteric peristalsis was also studied before division and re-anastomosis. These experiments are included among those described in chapter IV. In the remaining six experiments the ureters were divided straight away. The ureter was transected at a distance of 16–46 mm from the tip of the ureteric catheter

similar circumstances ICHIKAWA and IKEDA (1960) also made similar observations on isolated guinea pig ureters They obtained a propagation velocity in the action potentials of 24 1 $\pm$ 9 1 mm/sec at 32°C The peristaltic frequency probably also plays a part ICHIKAWA and IKEDA and also SLEATOR and BUTCHER (1955) stated that the propagation velocity was lower if a stimulus reached the ureteric muscles during a relative refractory period

That the velocity can vary between different parts of the ureter was also stated by GRAVES and DAVIDOFF (1923) In rabbit ureters they found a contraction velocity of 18 3 mm/sec in the upper third, 15 4 in the middle third and 13 3 mm/sec in the lower third Whether there was any definite difference in velocity in different parts of the ureter in the present material cannot be determined, since the diameter recording is not a sufficiently exact method for determining small differences

Results

The method used involved considerable difficulties with regard to the maintenance of a satisfactory blood supply to the distal ureteric stump It was at times impossible not to damage the arterial blood supply and in several cases ligation of the vessels on anastomosis sooner or later produced venous stasis in the distal stump as evidenced by swelling and signs of increased stiffness of the walls The period of observation in which conditions were favourable was thus in these cases relatively short (15–42 minutes) while in the remaining experiments the duration of the experiment could be extended to one hour or longer In four experiments the peristalsis could be observed for two hours or more after anastomosis In three cases the experiment could not be completed either because of inactivity in the whole ureter or because of technical failures

Variations in pressure and diameter in anastomosed ureters

In altogether thirteen experiments satisfactory observation conditions were obtained and peristalsis and contractility were observed in both ureteric stumps In seven of the cases recordings were also made before the ureter was divided

The pressure and diameter variations in contraction waves were in principle the same as in the experiments described in chapter IV (p 43) Thus as a rule a ureteric dilatation corresponding to a rapid moderate pressure increase (bolus jet wave) was seen before each contraction followed by a more protracted pressure wave If the ureteric pressure did not exceed 10 mm Hg the ureteric diameter diminution corresponding to the contraction appeared to be rather weak or absent The action potentials occurred during the dilatation phase at the passage of the bolus jet wave and had the same appearance as described previously In seven of the thirteen anastomosis experiments two or three bolus jet waves appeared in succession in the distal ureteric stump before its contraction The bolus jet waves always occurred with dilatations distally In the proximal segment they were synchronous at first with the dilatation at one or both

and the blood vessels were ligated A short polythene catheter, varying in length between 15 and 20 mm and with an external diameter suited to the lumen of the ureter, i e such that no distension or constriction should occur, was inserted *with one end* in each ureteric stump, and secured with ligatures The sectioned surfaces were not allowed to touch one another, but were isolated by a piece of rubber which had been threaded on to the anastomosis catheter before insertion Two diameter indicators of the type previously described were placed proximally to the anastomosis the distance between them varying between 9 and 25 mm and that between the more distal indicator and the anastomosis varying *between 10 and 21 mm A platinum electrode (see page 25)* was inserted intramurally into the distal ureteric stump 6–24 mm from the anastomosis, and a third diameter indicator was placed at the same level or very close to it The technique for the recording of electromyograms was the same as described in chapter III In some of the cases in which the peristalsis was studied before division, it was possible to perform the anastomosis without displacing the electrode or the diameter indicators The recordings before and after anastomosis were *therefore made* from exactly the same levels In one case only two diameter indicators were used, one being placed proximally to the anastomosis and the other distally at the site of the electrode

The aim of the experiments was to study the peristalsis after *total occlusion of the ureters* in particular at intraureteric pressure *levels between 0 and 20 mm Hg When the diuresis was* poor with an insignificant or absent pressure rise after ureteric occlusion, intravenous *injections of 0 9% sodium chloride solution* were administered to increase diuresis

For simultaneous intermittent recordings of ureteric diameters pressure and electromyograms a Visicorder type 906 A 1 was used The D C amplifiers used both for the diameter indicators and for the ureteric pressure transducer (Elema EMT 472 a) were Grass Low Level Preamplifiers In between the periods when recordings were made with the Visicorder the electromyogram was followed *continuously and the diameter changes in the distal* ureteric stump intermittently on a two channel oscilloscope A polaroid camera was used for photographing the oscilloscope picture

of the two diameter indicators later they also came with the contraction somewhere in the proximal stump The bolus jet thus appeared to be able to affect a large part of the ureter simultaneously The jet wave extended further in an anastomosed ureter than in an intact ureter the apparent reason for this being that fluid transport is facilitated in the permanently open anastomosis tube The bolus jet waves increased in amplitude with decrease in the distance between the site of contraction and that of the pressure measurement The action potential appeared during the last or in rare cases the penultimate wave Fig 13 shows the ureteric activity before (13 *a*) and after (13 *b*) anastomosis Before the transection the contractile frequency of the proximal part of the ureter was twice that of the distal part This may possibly have been due to insufficient stimulation (less pronounced dilatation phases) every alternate period or to the fact that distally the ureter was refractory After anastomosis the frequencies were the same in both parts All contractions were propagated and each complex included a small and a large bolus jet wave and analogous dilatation phases (13 *b*)

The contraction frequency at low intraureteric pressures

It was a general condition that with low intraureteric pressures a sufficiently large bolus jet wave and/or dilatation had to appear in the distal ureteric stump to provoke a contraction there If the contractions proximal to the anastomosis only gave small bolus jet waves or moderate dilatations of the wall no contractile response was obtained In nine experiments with basal pressures between 0 and 10 mm Hg a pressure wave of sufficient magnitude was obtained at each proximal contraction to provoke contraction in the distal stump The peristaltic waves were thus seen to propagate unhindered over the anastomosis (Figs 14 and 15) Fig 14 shows the recordings from intact ureters Dilatations and also very large bolus jet waves were observed at all measuring sites before the contractions Fig 15 shows the sequences from the same experiment after anastomosis lower frequencies and marked respiration dependent artifacts are seen

In four cases the frequency was higher in the proximal part and contractions occurred distally only after large bolus jet

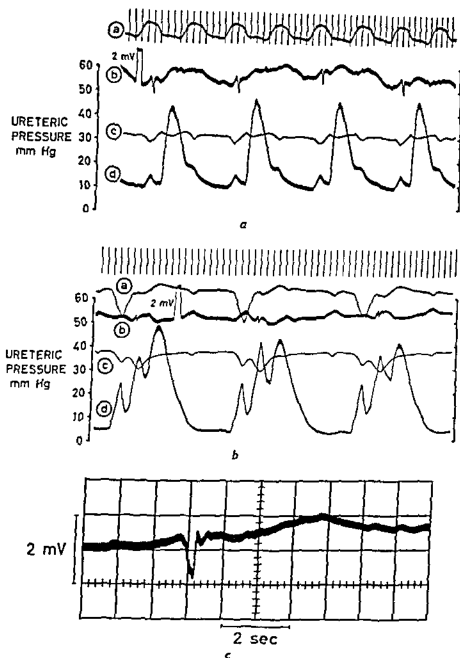


Fig 13 Ureteric contractions before (13a) and after (13b) transection and re anastomosis In both figures time in seconds is indicated uppermost and then the proximal ureteric diameter (a) electromyogram (b) distal ureteric diameter (c) and ureteric pressure (d) (b) and (c) were recorded at the same level Note that in 13a each proximal contraction results in a distal dilatation and rise in pressure but only each alternate proximal contraction is followed by a distal contraction In 13b the basal pressure in the ureter is lower and thus the contractions are not so marked There are respiration artifacts in (a) (b) and (c) in Fig 13b Recorded with a Visicorder Fig 13c shows an action potential recorded with an oscilloscope somewhat later than 13a

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In four cases the frequency was higher in the proximal part and contractions occurred distally only after large bolus jet

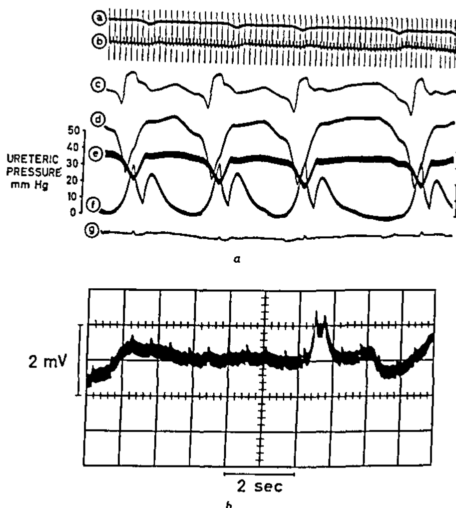


Fig 14 Peristalsis in a ureter with intact pelviureteric connections From above the time marking in seconds respiration (*a*) arterial pressure (*b*) (mean pressure 150 mm Hg) *diameter recording from three sites* (*c*) nearest to the kidney (*d*) 23 mm distal to (*c*) and (*e*) 38 mm distal to (*d*) ureteric pressure (*f*) and *electromyogram* (*g*) (*e*) and (*g*) were recorded at the same level The appearance of the action potential can be seen best on the oscilloscope picture (14*b*) The regular small disturbances in the electromyogram are due to a superimposed electrocardiogram

waves and/or dilatations Intermittently the contractions were propagated so that the frequency ratios were 2 1 3 1 or 4 1

The contraction frequency at high intraureteric pressures

At high basal pressures (15–30 mm Hg) the correlation between the activity in the two ureteric segments obviously dete

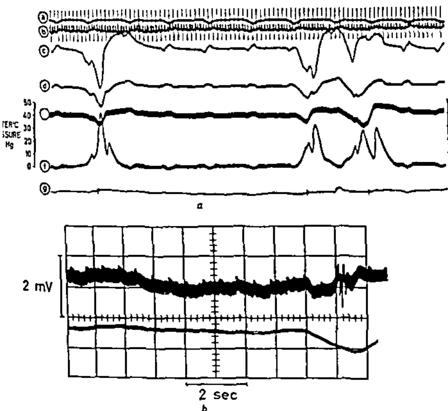


Fig 15 The s me expe iment as in F g 14 after transection and re-anastomosis betwe n (d) and ( ) The notat on is as in F g 14a The oscilloscope p cture (15b) shows the electromyogram (abo e) and diameter measurements from site (e) It is evident that the action potent al appears during a d latat on phase The electrocardiogram is upe imposed The second contraction in F g 15a seems to arise from a s te near the anastomo s [late d latation phase in cur e (c)] while the th rd contraction starts proximal to (c)

riorated and the distal stump functioned more or less inde pendently Both high and low frequencies were noted in the distal part but lower frequencies were more predominant than in the proximal region A proximal contraction was still able however to function as a trigger for the distal part (Fig 16)

At high basal pressures a volley of contractions was sometimes provoked distally A new action potential appeared here at the end of a contraction and at the end of the new contraction a further action potential appeared etc Every pressure wave how

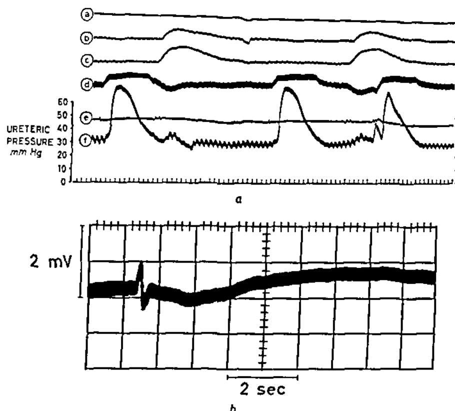


Fig 16 The activity in a re anastomosed ureter with high intraluminal pressure In Fig 16*a* are seen from above respiration (*a*) proximal diameter (*b*) diameter just above anastomosis (*c*) and below anastomosis (*d*) electromyogram (*e*) and ureteric pressure (*f*) Below time in seconds There are three contractions in the lower segment but only two in the upper During both (retrograde?) contractions in the upper segment the lower segment dilates and the pressure rises But only with the second contraction is the stimulation adequate to excite the lower segment Fig 16*b* shows the oscilloscope picture of a simultaneous action potential

ever, had a lower amplitude than its predecessor and finally the contractions faded out

Signs of retrograde peristalsis were observed on several occasions In particular a stimulus wave seemed to be able to turn at the anastomosis, but antiperistalsis produced from the distal part could also occur after a normal peristaltic wave had turned at the distal ureteric catheter

The velocity of the contraction waves

The propagation velocity was similar to that found earlier but varied over a somewhat greater range ie 9–24 mm/sec

instead of 12-20 mm/sec As a rule the velocity between the proximal and intermediate indicators appeared to be the same as between the intermediate and distal indicators (across the anastomosis) Occasionally propagation was more rapid across the anastomosis. Velocity determinations could only be made when clear contractions were recorded at all measuring points This required an intraureteric pressure of more than 10 mm Hg which was reached in only eight cases At low pressures the contractions are not manifested as diameter reductions This has been discussed in more detail in chapter IV

Discussion

The regulation of ureteric peristalsis

The question of the nature of propagation in smooth muscle has been extensively investigated Different types of organs have been studied (oesophagus taenia coli uterus vascular walls ureter *inter alia*) with different techniques Opinions have varied regarding the importance of an autonomous innervation in the case of the ureter but the majority oppose the view that ureteric peristalsis is governed or influenced by autonomous nerves (ENGELMANN 1869 BOZLER 1938 DAVALOS 1947 LAPIDES 1948 MORALES *et al* 1952 RISHOLM 1954 SLEATOR and BUTCHER 1955 COREY *et al* 1956 O CONOR and DAWSON EDWARDS 1959 O CONOR 1961 MELICK *et al* 1961 *b* HAMM and WEINBERG 1962) There have been some dissident voices however BAKER and HUFFER (1953) and GOULD *et al* (1955 *a*) considered that the ureter had a double autonomous regulation in which the sympathetic impulses decreased and parasympathetic impulses increased the motility or tone HANLEY (1953) reported in clinical material that spastic ureteritis showed improvement after denervation SATANI (1919 *a*) and DURAND and DESCOTES (1952) demonstrated certain effects of nerve stimulation on the ureter while KHARITONOV (1957) claimed he was able to inhibit ureteric peristalsis neuroreflexly There seem to be no advocates at present for the theory of a *humoral* regulation of the ureter The question of mechanical extension or stimulation from cell to cell has been discussed with great interest and evidence for the occurrence of

both types of stimuli has been claimed It has often been postulated that the smooth muscle cells in the ureter form a sort of syncytium *It has also been suggested that an excitatory stimulus* passes between the cells over intercellular bridges (BAKER and HUFFER 1953) BERGMAN (1958), in an electron microscopic investigation of the ureter in white rats, found intercellular bridges, which he believed could transmit stimuli between the cells The smooth muscle of the ureter would thus constitute a *functional* syncytium (BERGMAN 1958) The modern theories for conduction are discussed in more detail in chapter VI

The results obtained from investigations of smooth muscle do not provide an unequivocal answer to the question regarding the propagation of stimuli, but the microscopic structure of the ureter seems to provide a possible basis for electrical activation from cell to cell *The present investigation shows, however, that mechanical stimulation alone is sufficient to propagate a peristaltic wave over an interruption in the continuity of the ureter* There seems further, to be a relationship between the magnitude of the stimulus and the response produced, since a stimulus that is too small results in no contraction This conclusion has been drawn previously (PROTOPOPOW 1897, ANDERSON 1951 *inter alia*) The fact that a peristaltic wave can fade out in the ureter can also be explained in this way This has been especially observed where a pool has formed in the ureter and where an incoming urine bolus is not always able to provoke a contraction but where the whole pool sometimes empties (GOULD *et al* 1955 *b*) KIIL (1957) described similar findings in man He considered it possible that the contraction in a pool differs from an ordinary peristaltic wave, and that a distension of the pool can provoke a contraction that empties the whole pool MURNAGHAN (1958) also held similar views The ureter regarded as a unit, should thus be able to function according to the all or none law although the individual muscle cells probably do not function in this way

The peristalsis in anastomosed ureters

In other investigations also with anastomosis of the ureter the results seem to fit in with a hypothesis that conduction is de

pendent on mechanical stimulation If an adequate healing time is taken into consideration the results have shown that propagation can occur unhindered over an anastomosis WEINBERG and SIEBENS (1958) in electromyographic studies of the peristaltic frequency before and after transection and end to-end anastomosis found a considerably lower frequency distally immediately after the anastomosis had been performed On examination 4–8 months later no obvious difference was seen and neither was there any retardation across the anastomosis itself as BUTCHER and SLEATOR (1956) claimed after similar experiments The reason for the lower ureteric activity immediately after transection must be due at least partly to non specific factors e g tissue oedema haematoma and obstruction which prevent the passage of urine and render impossible adequate distension distally The importance of careful surgical technique to avoid strictures was pointed out by DAVALOS (1947) among others The frequently observed finding of moderate hydronephrosis proximal to an anastomosis during the first 2–3 weeks post-operatively before the return of adequate peristalsis may possibly be due to mechanical obstruction during the healing period The role of stenosis in the occurrence of hydroureters was discussed by ISELIN (1929) who considered that *the stenosis and not the discontinuity in the musculature was the deciding factor* Mobilization of the ureter has also often been described as sufficient to temporarily inhibit the ureteric activity and may probably contribute to an initial reduction in peristaltic frequency

WEINBERG and SIEBENS attempted to explain the retardation across the anastomosis found by BUTCHER and SLEATOR in their chronic experiments by differences in surgical technique There was also another difference BUTCHER and SLEATOR stimulated the ureter with an intraluminal balloon and there was no fluid available to distend the ureter distally to the anastomosis It seems plausible that in a scarred and empty ureter with muscular discontinuity the contraction wave will be delayed in the region of the scar If there is however a fluid bolus advancing in front of the contraction wave this should be able to activate the muscles on the other side of the scar, thus perhaps eliminating the contraction delay

BENJAMIN *et al* (1956) carried out a series of anastomosis ex

periments with a polyethylene tube 5 cm long A defect was either bridged with the tube alone or half of the circumference of the ureter was retained After healing, contractions were observed both above and below the defect in both types of experiment No synchronization was found, however, between the two parts of the ureter If the conduction were purely myogenic, at least some of the contractions should have been propagated in those cases where half of the ureteric circumference remained Experiments with transsectioning of half of the circumference were also reported by ALKSNE (1907), who found that a contraction ceased at the site of incision if the latter extended into the lumen but continued unhindered if the mucosa was intact WEINBERG (1962) made a corresponding observation on the relationship between flow and activity in studies of the peristalsis after diversion of urine through a vertical ureterotomy WEINBERG recorded electromyograms both proximally and distally to the ureterotomy and found that action potentials appeared only proximally, while the distal part, to which no urine penetrated remained inactive WEINBERG found a return of normal peristalsis after healing If the conduction takes place only by means of myogenic spread a linear vertical ureterotomy should not have a blocking effect

The effect of dilatation proximally to a stenosis

In the anastomosis experiments of BENJAMIN *et al* described above, a dilatation was recorded in the upper ureteric segment BENJAMIN considered that there was flow resistance in the rather narrow prosthesis *It is conceivable that in situations with ureteric dilatation proximally to a stenosis or other forms of flow impediment a damping occurs such that with the proximal contractions a bolus jet wave sufficiently large to excite the musculature does not reach the post stenotic region* Perhaps not until a certain degree of fluid filling occurs in the distal segment is there sufficient distension to provoke a contraction An apparent inactivity might thus be due to mechanical factors and the ureter would be assessed as atonic since the proximal contractions were masked by damping and the distal contractions were only seldom provoked

The importance of regenerative power to ureteric function after anastomosis

The ureteric muscles have been considered to have good regenerative powers, especially if there is some form of contact between the cut surfaces. The good results obtained after long periods of observation could therefore be due to complete healing in the operation region but good propagation has also been demonstrated when prostheses separate the sectioned surfaces. Studies of the power of regeneration in the ureter were made by OPPENHEIMER and HINMAN (1955) and HINMAN and OPPENHEIMER (1956) *inter alia*. They found good healing in parts where half of the circumference had been excised and it was best if no splint had been used. The mucosa regenerated within a week and the smooth muscle within 4–6 weeks. The flow of urine through the operation region delayed healing. Similar results were obtained by DENIZ *et al.* (1962) who found good but not complete healing of the circular musculature and also that free urinary flow could cause extensive fibrosis. HAMM and WEINBERG (1956) performed experiments with linear ureterotomy and other longitudinal defects and also found good healing. KOČVARA and ZAK (1962) obtained good healing in dog ureters with teflon prostheses and in cases in which a central ureteric segment had been substituted a normal ureteric passage was also evident on excretoric urography after transient reduction in function and stasis as well as normal ureteric dimensions both above and below the prosthesis.

In man observations have been made mainly during operations on hydroureters. MELICK *et al.* (1962) observed that with low pressure and pronounced dilatation human ureters after transplantation from bladder to skin re-acquired normal size and regained relatively normal intraureteric pressure in a number of cases. SWENSON and SMITH (1959) made similar findings on children with megaloureters. After cutaneous ureterostomy aperistaltic ureters to a large extent regained good peristaltic function. The likelihood of this occurring can depend on the pre-existing degree of ureteric enlargement (SWENSON *et al.* 1959).

In spite of the good power of regeneration in the smooth muscle of the ureter complete healing has never been described. According to ISELIN (1929) and others this is not however

necessary for good peristaltic function The results of previous investigations with prolonged experiments without more severe grades of stenosis should therefore, in principal, be similar to those of the present acute experiments Difficulties with regard to the blood supply in these latter experiments seem to have as their counterparts the stenosis, dilatation and other disturbances in short term chronic experiments The circulatory disturbances as a rule probably have only a transient effect on ureteric function, even if in acute experiments they can render the observations impossible The blood vessels running along the ureter should, however, be adequate for the nutrition of a stripped or transplanted ureter (O'CONOR 1961)

Changes in function due to transection

In the anastomosis experiments the principal findings were the same as with intact ureters (chapter IV) *It is clear that depolarization in the muscle cells occurs at the same time as and is dependent on the distension, regardless of whether the peristaltic propagation is provoked mechanically and/or syncytially* The trigger effect represented by the extension of the ureteric wall must be sufficiently large on anastomosis to prevent a peristaltic wave from fading out Whether a presumptive propagation from cell to cell changes the sensitivity to the trigger effect and thus an anastomosis would reduce the excitability cannot be determined from the present study In most cases the lower segment had the same peristaltic frequency as the upper, both before and after transection and anastomosis in other cases the activity was reduced distally after the division Operational trauma certainly plays some part but it is highly probable that the ureter functions more satisfactorily if there is physiological contact between the muscle cells An example of this was seen in the perfusion experiments on rabbits discussed in chapter II where the peristalsis deteriorated considerably when the ureter was divided at the renal pelvis The sensitivity can probably vary both between different parts of the ureter and between different animal species

The passage of fluid through the anastomosis tube

The insertion of an anastomosis tube that keeps a part of the ureter open even when no fluid is distending the lumen can

have several effects As mentioned in chapter II the local static tension in the wall at the place of insertion of the tube might be expected to result in the appearance of spontaneous propagated contractions even at low intraureteric pressure levels This was not observed in the present series of experiments An open communication should allow more rapid passage of fluid and at the same time result in a pressure effect over a larger part of the ureter than when the urine bolus itself has to open the lumen as it progresses along the ureter The existence of several bolus jet waves preceding a contraction showed that the latter effect occurred Each bolus jet wave was more extensive and a summation was obtained in certain cases with 2-3 successively higher pressure steps in the ureteric pressure recording The correlation between the proximal contraction and the passage of the dilatation and bolus jet wave distally was good and well illustrated the origin of this pressure increase

As regards the more rapid fluid transport through an open tube there was a greater velocity of the bolus front across the anastomosis in a few experiments The anastomosis tube however cannot be dilated by the passage of fluid and if a large bolus is to be pressed forward it is not certain that the open rigid connection has always a lower resistance to flow than an elastic walled organ which is initially closed Furthermore the number of observations giving indirect information on this point was very small and the sensitivity of the diameter indicators somewhat inadequate so that with regard to the divergent results no definite conclusions can be drawn

At high intraureteric pressures the correlation between the contractions on either side of the anastomosis deteriorated The two ureteric stumps contracted to a large extent independently of one another and it would seem reasonable to assume that the intraureteric pressure was the provoking factor If the prevailing conditions are sufficient for stimulation no extra pressure increases are required in the form of a bolus jet wave to provoke activity and the frequency then becomes dependent on the strength of the pressure stimulation and the condition of the ureter In an intact ureter however it is not presumably advantageous to have different frequencies in different parts of the ureter As has been described in chapter III no frequency

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CHAPTER VI

The conduction mechanism in the smooth muscle of the ureter

As is evident from the discussions in chapters IV and V there is some disagreement as regards the conduction mechanism in the ureteric musculature No general rules for conduction in smooth muscle can be laid down since different types of smooth muscle show obviously different patterns of behaviour BOZLER (1948) classified smooth muscle into two groups multiunit muscles in which the conduction was assumed to be transmitted by nerves and unitary muscles in which the conduction was said to take place by the spread of stimuli from muscle fibre to muscle fibre The former category includes for example the pupillary ciliary pilomotor and vascular muscles There is some disagreement however concerning the conduction in the muscles of the vascular walls (BURNSTOCK and PROSSER 1960 *a* RODDIE 1962 *a* and *b*) The visceral muscles are included in the unitary group but they are not however an entirely homogenous group Thus for example the excitability of the uterine musculature is affected by some hormones and the intestinal and the ureteric muscles react differently both to the influence of some drugs and to nervous stimulation Because of the differences between the various types of muscle therefore the following discussion will mainly be limited to the ureter

Theories regarding the conduction mechanism

According to the four principal current theories conduction can be mediated by (*a*) nerves (*b*) transmitter substances liberated between the muscle cells (*c*) electrical current spread from cell to cell or (d) mechanical distortion

(*a*) *Nerves* While in older investigations it was considered as a rule that the ureter was governed by nerves it is now generally

difference was found in the experiments with high pressure and free pelviureteric communication in the few cases where several diameter indicators were used. *The fact that the frequency is the same at different levels in an intact ureter is probably due to the fact that somewhere in the musculature there is a pacemaker, the frequency of which dominates the entire organ by the physiological connection between the muscle cells.* If the ureter is divided by an anastomosis the muscular connection is interrupted, and an impulse emanating from the pacemaker cannot directly bridge the anastomosis. If the ureter is dilated and the intraluminal pressure high, a functional pacemaker may then be present in each part of the ureter. It is highly probable in this case that the frequencies differ, since the spontaneous contraction frequency has been found to be different in different parts of the ureter (SHIRATORI and KINOSHITA 1961 *b*, and others). In agreement with this, different frequencies in both parts of the ureter were also recorded in the present anastomosis experiments with high pressures.

As in the series of experiments described in chapter IV signs of antiperistalsis were observed. No decisive evidence of this was obtained, however, since neither electromyograms nor pressure were recorded in the upper part of the ureter. In experiments with pig ureters, however MELICK *et al.* (1961 *a* and *b*) showed that normal peristaltic propagation could be obtained in a ureter even when a segment had been excised, subsequently reversed in direction and re-anastomosed. Thus in the transplanted segment the contractions moved in an opposite direction to the pre-operative contractions in that segment and the ureter functioned well. *For a definite solution of the problem regarding the occurrence of antiperistalsis under different conditions, further investigations are required with measurements at several points of the electromyogram, diameter and pressure.*

muscles constitute a syncytium (ENGELMANN 1869 BOZLER 1935) or the stimuli are transmitted through the intercellular space to adjacent cells In this case the impulse might either pass over intercellular bridges (=functional syncytium) via overlapping muscle fibers (=ephaptic conduction) or through non excitable material The hypothesis that some form of electrical conduction occurs has many advocates (PROSSER *et al* 1955 BULBRING *et al* 1958 ICHIKAWA and IKEDA 1960 and others) PROSSER *et al* (1960) showed in comparative studies that there was a clear relationship between the size of the intercellular space and the conduction velocity The greater the distance between the individual muscle fibres in an organ the lower was the conduction velocity observed The excitation should thus be propagated more rapidly if the distance between the cells is small

Like other visceral musculature the ureteric muscles exhibit two types of membrane potential variations Both slow small potential changes and spike potentials have been shown The former are accompanied by tone changes in the muscle cell (BOZLER 1942 and 1948) the latter by regular contractions The slow potentials are propagated only to an insignificant degree and have been related to a pacemaker function (BOZLER 1942 ICHIKAWA and IKEDA 1960 BURNSTOCK and PROSSER 1960 *b* BULBRING 1961 and others) There appears to be no clear idea of the optimal contraction frequency of the ureter In consideration of the long absolute refractory period of the ureter (1–3 seconds) the characteristic frequency should be fairly low and it seems in fact to vary within broad limits Experiments with electrical stimulation reported up to the present time have not cleared up this problem fully PROSSER *et al* (1955) found that the spontaneous rhythm in the ureter varied and was sometimes higher than that to which the ureter could be driven electrically The highest frequency that PROSSER could get a rat ureter to follow was about 1 c s

(*d*) *Mechanical distortion* There seems to be no doubt that an increase in tension in the ureter is an adequate stimulus for initiating contraction A large number of investigations agree on this point Intraluminal balloons and injections of fluid and also pinching and stretching have been used The same response was produced regardless of the manner of distension Isolated prepara

held that the ureteric musculature is fairly autonomous The "extrinsic" nervous influences on ureteric peristalsis have been discussed in chapter V As regards the hypothesis that *conduction* is mediated by nerves, this is clearly not supported for example, by the low propagation velocity and low sensitivity to nerve and synaptic blocking drugs (Bozler 1938, Lapides 1948, Sleator and Butcher 1955, Prosser *et al* 1955, Borgstedt 1960 Mazella and Schroeder 1960) Nerve tissue does exist in the ureter but its role in normal peristaltic function is still unclear

(*b*) *Transmitter substances* The question of a transmitter substance has been much discussed in the literature, and attempts have been made to demonstrate its existence The role of histamine in ureteric function has attracted particular attention (see Borgstedt *et al* 1962 for further references) Butcher and Sleator (1956), in experiments with re anastomosed ureters, found a delay in conduction across the anastomosis, which they considered was possibly due to the slowness of penetration of a presumptive transmitter substance through the scar tissue Butcher *et al* (1957) also came to similar conclusions

The occurrence of cholinesterase in the urine has aroused especial interest Benjamin *et al* (1956) found higher concentrations of the enzyme in dogs than in man but no difference between normal and pathological ureters in the same species de Klerk (1954) also studied the cholinesterase in dog ureters and found that perfusion with a cholinesterase inhibitor could abolish peristalsis He discussed the existence of an inhibitory substance in the urine and its possible significance in the occurrence of ureteric structures

(*c*) *Electrical current spread* A large number of investigations have been performed especially with isolated preparations and varying electromyographic techniques in order to shed light on the problem of whether conduction takes place by means of the transmission of action currents from cell to cell Several workers have shown that electrical stimulation of the ureter provokes contractions (e g Engelmann 1870 Bozler 1938 Sleator and Butcher 1955 Ichikawa and Ikeda 1960) It seems reasonable, also, to suppose that the cell membranes might be depolarized by an electric shock

Two types of transmission are possible Either the ureteric

muscles constitute a syncytium (ENGELMANN 1869 BOZLER 1938) or the stimuli are transmitted through the intercellular space to adjacent cells In this case the impulse might either pass over intercellular bridges (= functional syncytium) via overlapping muscle fibers (= ephaptic conduction) or through non excitable material The hypothesis that some form of electrical conduction occurs has many advocates (PROSSER *et al* 1955 BULBRING *et al* 1958 ICHIKAWA and IKEDA 1960 and others) PROSSER *et al* (1960) showed in comparative studies that there was a clear relationship between the size of the intercellular space and the conduction velocity The greater the distance between the individual muscle fibres in an organ the lower was the conduction velocity observed The excitation should thus be propagated more rapidly if the distance between the cells is small

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tions also react in a similar way. Several authors have shown that there is a relationship between the degree of tension and the membrane potential in a muscle cell (BULBRING 1955, BURNSTOCK and PROSSER 1960 *a*, GILLESPIE 1962 *a* and *b*). Increased stretching gives (sometimes after an initial, single spike) increasing depolarization, and *with sufficiently high excitability the cell discharges*, a spike potential occurs and contraction follows. GILLESPIE (1962 *a*), in experiments with colon muscle, found a depolarization corresponding to 5.5 mV when the preparation was only lightly stretched. This difference from the resting potential in an unstretched state was stated as being statistically highly significant. BURNSTOCK and PROSSER (1960 *a*) found the same order of magnitude on depolarization with rapid stretching of the ureter in rats and guinea pigs. Guinea pig ureters responded to an extension of 1 mm per 12 mm muscle length. BOZLER (1947) investigated the effect of stretching in dog ureters and found that distension above a certain limit provoked slow transient potential changes. *The response was related to the distension applied*. On further stretching a conducted response arose from the local response.

There thus appears to be a distinct relationship between the degree of stretching in the muscle cell and membrane polarization *and also therefore the excitability, but the rate at which an increase in tension occurs is probably important*. In model experiments with a pressure-sensitive cell analog which was subjected to one particular pressure rise but initiated with different velocities, TEORELL (1959) found that a certain minimal velocity *in the pressure increase was required to obtain a potential response*. Static and dynamic pressure in the carotid sinus and also the firing frequency of potentials in the carotid sinus nerve were studied by WARNER (1958) who formulated a relationship between these factors.[1] TEORELL (1962) examined this relationship

[1] WARNER used an electronic analog of the carotid sinus and the following equation was used to describe its function

$$n = K_1 (P - P) - K_2 \frac{dp}{dt}$$

where n is the frequency of action potentials at a point on the carotid sinus nerve, P is the pressure in the carotid artery, P is the minimum *static pressure* capable of eliciting action potentials and dp/dt is *the rate of change of* pressure in the artery. K_1 and K_2 are constants.

with an analog computer and found that apart from the application of a minimal pressure a certain velocity in the pressure increase (dynamic pressure) was also required to initiate a potential spike High pressure applied with a velocity that was too low could thus be insufficient while a lower pressure with more rapid rise sufficed to give a potential spike One of the aims of the perfusion experiments described in chapter II was to investigate these circumstances in biological material The velocities chosen here however produced no differences in response which must mean that both lay above the critical threshold BURNSTOCK and PROSSER (1960 *a*) however observed differences in the response between rapid and slow stretching of smooth muscle

It is thus clear that increase in tension in the ureter gives rise to increased excitability Conduction might therefore occur by the mechanism that one cell in contracting distorts an adjacent cell sufficiently for this cell in its turn to be depolarized It is also possible that the intraluminal rise in pressure occurring distally to a contraction acts as a depolarizing extension (GOULD *et al* 1955 *a* HAMM and WEINBERG 1962 and others) Ureteric muscle contractions without simultaneous fluid transport have been described however by several workers and the passage of urine therefore does not seem to be necessary but is probably responsible normally for a very important part of the distension The experiments described in chapters IV and V gave clear evidence that adequate distension was required to prevent the peristaltic wave from fading out The importance of mechanical distortion in the conduction in smooth muscle has been questioned (SPERELAKIS and PROSSER 1959 BURNSTOCK and PROSSER 1960 *b*) The knowledge of the effect of distortion on the excitability makes it difficult however to minimize the importance of the stimulus produced by an extension caused by contraction of the adjacent cells The bridges observed between the ureteric muscle cells (BERGMAN 1958 PROSSER *et al* 1960) might in consideration of the lack of protoplasmic continuity between the cells have a high electrical resistance It must therefore be asked whether such connections can in fact constitute a transmission path for action currents It is not precluded that these bridges may be purely mechanical connecting link (PROSSER *et al* 1960)

The relationship between electrical transmission and mechanical distortion

It must be considered proven that electrical transmission can take place between the cells. It is reasonable to assume, however, that it is not solely responsible for the conduction in the ureter. The phenomena of frequency differences, pool formation and inactivity at low pressures support this hypothesis. An electrical impulse alone often seems to be insufficient to depolarize the cell membranes to such a degree that a spike potential occurs and the contraction wave then dies out. *For the persistence of normal propagation, therefore, a simultaneous mechanical component is probably required.* This mechanical component involves an adequate degree of stretching, provided this stretching takes place at a sufficiently rapid rate. The degree of stretching caused by a given stimulus (a urine bolus or stretching from the adjacent cells) is dependent on the tone of the individual ureter and its power to change the volume for a given tension increase. That which may be an adequate stimulus in one case can possibly be below the stimulation threshold in another. The magnitude of the static pressure induced seems to be of the greatest importance here, since the perfusion experiments described in chapter II allow us to suppose that the threshold rate of the pressure increase does not need to be fast and is probably usually attained.

The view discussed above is similar to that of BURNSTOCK and PROSSER (1960 *b*). They concluded that propagation in smooth muscle depends on more than one factor. They considered that the transmission seems to be electrical but propagates best if the muscle has become excitable by stretching or chemical transmitters. The results of the present experiments are not in disharmony with this view. The required excitation is apparently obtained by distension provoked by the bolus jet (or at higher intraluminal pressures by the prevailing tension in the wall). Under special conditions—the anastomosis experiments—it was shown that the distension alone without obvious delay was capable of provoking a continued contraction wave. The fact that direct muscular continuity normally plays a part in the propagation, however, was indicated by the lack of contraction asynchrony at

high intraureteric pressures when the ureter was intact whereas on the other hand different frequencies were recorded in the different segments after anastomosis *The regular activity in the intact ureter could thus be due to spike potentials, emanating from a pacemaker somewhere in the organ and propagated along the ureter as long as this is made excitable by adequate distension*

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There was no difference in the stimulation effect between the two rates of pressure increase used

The contractile power at intraureteric pressures higher than 15-20 mm Hg was studied by increasing the perfusion pressure up to a maximum of 155 mm Hg *Pressure variations due to contractions were shown up to 125 mm Hg* The degree of damping in the perfusion system could be decreased intermittently, the amplitude of the pressure waves thereby always increasing It was concluded that *if a ureter with intact connection with the pelvis dilated after obstruction to such an extent that open fluid communication with the pelvis and therefore regurgitation occurs, the peristaltic pressure waves are damped considerably* In the perfusion system used the degree of pressure damping was fairly low and the pressure waves could therefore be identified even with very high intraureteric pressures

Chapter III The contractility at high intraureteric pressures with intact pelviureteric connections

Ureteric peristalsis was studied in a series of experiments with intact pelviureteric connections The intraluminal pressure was measured distally after total occlusion, and the electromyogram and ureteric diameter just proximal to the tip of the ureteric catheter were recorded at the same time On increase of the basal pressure in the ureter the character of the pressure waves changed and when there was open fluid communication with the pelvis the only important pressure oscillations observed were synchronous with the arterial pulse Action potentials and ureteric contractions accompanied the peristaltic waves at lower intraluminal pressures The action potentials and ureteric contractions did not change in character when the pressure became so high that the damping in the pelvis eliminated the pressure waves The contraction frequency however showed a slight tendency to decrease No relationship between respiration frequency and ureteric activity was shown It is concluded that *the earlier views that the ureteric contractions cease on high pressure are dependent on the fact that neither pressure measurement alone nor contrast roentgenography is sufficient to demonstrate ureteric activity when the upper urinary tract is dilated*

General summary

The aim of the present work was to study the relationship between the intraluminal pressure and the contractility in the ureter Dogs were used for the experiments The effect of different high intraureteric pressures was studied on ureters *in situ* both with perfusion methods and with retention of the physiological connection between the renal pelvis and the ureter A series of experiments with transection and re anastomosis of the ureter was performed in order to study the propagation mechanism of the peristalsis

Chapter I Introduction

A short history of the physiology of the ureter is presented, and some general problems of interest to this investigation are mentioned

Chapter II Perfusion of the ureter in situ

For measurement of the intraluminal pressure a polythene catheter was inserted distally in the ureter close to the urinary bladder The ureter was totally occluded and the peristalsis was studied An attempt was made to obtain an intraluminal pressure of more than 25–30 mm Hg and diuretics were given when necessary With a progressively increasing intraluminal pressure (above 15–20 mm Hg) the contraction dependent pressure variations in the ureter decreased gradually and finally became immeasurably small In a second part of the experiments perfusion was carried out via a polythene catheter which was inserted into the ureter near to the renal pelvis There was thus no fluid communication between the pelvis and the perfusion system but the nervous and vascular connections between the pelvis and the perfused ureter were left as intact as possible The perfusion pressure was raised at two constant rates first at 0 97 mm Hg/min and subsequently at 6 05 mm Hg/min The ureteric peristalsis started at pressure levels between 1–2 and 16 mm Hg

is increased above 15–20 mm Hg spontaneous contractions occur in both parts of the ureter, but the distal part usually has a lower frequency

The regulation of ureteric peristalsis is discussed and the effect of an anastomosis examined Comparisons are made with anastomosis experiments previously reported The conduction mechanism in the ureter is discussed briefly and it is concluded that *mechanical distortion plays an important part as regards peristaltic propagation*

Chapter VI The conduction mechanism in the smooth muscle of the ureter

The modern theories on the conduction mechanism are reviewed Experimental data from studies of the ureteric muscles in particular are discussed It is concluded that the conduction mechanism seems to consist of two components (*a*) an electrical and (*b*) a mechanical one (*a*) *There is probably electrical transmission from cell to cell* (*b*) *A mechanical distortion of the muscle cells may be required to produce a sufficiently high degree of excitability making possible the electrical transmission* The way in which this distortion is produced in the ureter with normal peristalsis and at raised pressures is discussed

Chapter IV Ureteric diameter variations and electromyograms at low intraluminal pressure levels

Ureteric peristalsis was studied at pressures lower than 15-20 mm Hg and with the pelviureteric connections intact. The ureteric diameter was recorded at two or three different levels and action potentials were measured from a point somewhat proximal to the pressure measurement catheter which was situated distally. The pressure was measured after total occlusion. Diphasic pressure waves as a rule appeared with the peristaltic contractions. The first phase in each pressure wave coincided with a contraction at a more proximal measuring site, and was apparently due to a urine bolus pressed forwards. The forward penetration of a urine bolus distended the ureter, and at the same time an action potential appeared in the dilated region. The second phase in the pressure wave occurred when the muscle contraction reached the measurement site as manifested by a diameter reduction which started after the action potential. When the basal pressure of the ureter became higher the first phase in the pressure wave gradually decreased, and the simultaneous ureteric dilatation also decreased at the same time. The relationship between the pressure waves, diameter variations and action potentials are discussed and it is concluded that *the ureter probably has to be excited by a certain degree of stretching before a peristaltic wave is able to propagate.*

Chapter V The peristalsis in an anastomosed ureter

The same technique was used as described in chapter IV. The peristalsis was studied before and after transection and re-anastomosis with a short polythene catheter. The same peristaltic findings with diphasic pressure waves, dilatation-contraction and action potentials were made as described in chapter IV. *After anastomosis of the ureter it was shown that a peristaltic wave could propagate unhindered over the site of division even though there was no myogenous or nervous connection between the two parts of the ureter.* A prerequisite here is that a bolus jet (advancing in front of a contraction wave proximally) should induce a distension, distally to the anastomosis, sufficiently strong to stimulate contraction of the muscles. If the intraluminal pressure

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Aars Harald (Nuffield Department of Surgery Radcliffe Infirmary Oxford England and the Institute for Experimental Medical Research University of Oslo Norway) EXPERIMENTAL POST STENOTIC DILATATION OF THE ASCENDING AORTA IN RABBITS

Previous experimental studies of post stenotic dilatation have been hampered by unsatisfactory constriction techniques

A new method is described whereby acute constrictions of the intrapericardial part of the ascending aorta in rabbits are induced by a tapered nylon ring The inside diameter of the ring is known and the internal diameter of the aorta is measured on angiocardiograms taken prior to the opening of the chest This method thus provides opportunities for relating the eventual vascular and hemodynamic changes to a known degree of stenosis Directly under the ring the vessel wall will gradually get thinner but the rabbits have tolerated well a 70–80 per cent reduction of the inside cross sectional area of the aorta for 10 months

Provided a severe stenosis is present a post-stenotic dilatation is formed after a few days as illustrated by angiocardiography The dilatation is comprised of the rest of the ascending aorta the whole of the arch and the first few centimeters of the descending aorta Occasionally the adjoining parts of the cervico-brachial vessels are involved Histological examination reveals corresponding degenerative changes in the intima and media. In rabbits on a normal diet thrombi are not formed in the dilated area in any significant number

This experimental model is presently used in studying the effect of the dilatation on thrombi and cholesterol deposits in rabbits fed a high fat diet and the effect on the function of baroreceptors in the dilated aortic arch

Thus FSH stimulates the rate of protein synthesis in at least some of the ovarian cells an effect which might be due at least in part to a stimulation of the rate of entry of amino acids into the intracellular amino acid pool

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Ahren, Kurt and Lidia Rubinstein* (Department of Physiology, University of Goteborg, Sweden) STIMULATION OF GLYCINE 2-T-INCORPORATION INTO THE PROTEIN OF ISOLATED RAT OVARIES BY FOLLICLE STIMULATING HORMONE (FSH)

Injection of FSH into prepubertal rats in one intravenous dose 2–4 h before removal of the ovaries stimulated the accumulation of the non utilizable amino acid, α aminoisobutyric acid (AIB) in the ovaries (*Ahren and Kostyo* 1962, 1963) In the present experiments the effect of FSH on the incorporation of glycine 2 T into the protein of isolated rat ovaries and the accumulation of AIB C^{14} was studied

Ovaries from six prepubertal rats (24–26 days old) were pooled for each determination The ovaries were divided into two groups with one ovary from each rat in each group One group of six ovaries was incubated for 2 h in Krebs phosphate or bicarbonate buffer containing 0 1 mM glycine-2 T and 5 6 mM glucose, and the other group of six ovaries was incubated in a medium where glycine-2-T was replaced by 0 1 mM AIB C^{14} At the end of the incubation period the ovaries were homogenized in 10 per cent trichloroacetic acid (TCA) and the accumulation of soluble radioactive material was measured The protein was prepared as described by *Manchester and Young* (1958) dissolved in 1 M Hyamine and counted in a liquid scintillation spectrometer

With glycine-2-T in the medium the incorporation of tritium into the protein (calculated as cpm/mg protein) was significantly greater in ovaries from rats which had received one intravenous injection of ovine FSH (5 μg/g body weight) 4 h before removal of the ovaries than in simultaneously incubated ovaries from control rats In addition, the accumulation of TCA soluble radioactive material by the isolated ovaries was stimulated by the injection of FSH

As in the previous study (*Ahren and Kostyo* 1962, 1963) AIB C^{14} was not incorporated into the protein of the ovaries and injection of FSH 4 h before removal of the ovaries stimulated the accumulation of this non utilizable amino acid by the isolated ovaries

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graft In the rats with 4 grafts alveolar development was slightly stimulated by testosterone but alveoli were still scarce as compared with normal controls

Thus there is a deficiency of growth hormone in hypophysectomized rats also when these rats have 4 pituitary glands transplanted under the kidney capsule This indicates that a normal connection between the hypothalamus and the hypophysis is essential for the secretion of STH

Ahren K., Acta endocr (Kbh) 1962 *39* 338

Hertz R Endocrinology 1959 *65* 926

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Ahren, Kurt and Lidia Rubinstein* (Department of Physiology University of Goteborg Sweden) STUDIES ON GROWTH HORMONE PRODUCTION BY THE TRANSPLANTED HYPOPHYSIS

When the anterior pituitary gland is transplanted to a site in the body remote from the hypothalamus it does not produce significant amounts of follicle stimulating hormone, luteinizing hormone, thy roid stimulating hormone or adrenocorticotrophic hormone, but is able to secrete considerable amounts of luteotrophic hormone It is uncertain whether the grafts produce growth hormone (somatotrophic hormone STH)

The capacity of the rat mammary gland to respond to testoster one stimulation with lobule-alveolar development only when STH is present, was used to study the secretion of STH by the pituitary gland autotransplanted under the kidney capsule (*Ahren* 1962) These experiments indicate that there is a considerable deficiency of STH in rats with pituitary autografts The results, however could be attributed to the lack of sufficient functional tissue, since *Hertz (1959)* reported somatic growth at about two thirds of the normal rate in hypophysectomized rats bearing four whole pituitary glands under the kidney capsule It was therefore decided to compare the somatic growth and the mammary gland response to testosterone in rats with one and four hypophyses transplanted under the kidney capsule

Male rats were castrated at 4 weeks of age Three weeks later the anterior pituitary gland was transplanted and 3 days later one group received in addition 3 pituitary glands from male rats 4 weeks old

The body weight and length were studied for 15–17 weeks Dur ing this period the increase of body weight in rats with one trans plant was 6–20 per cent, in rats with 4 transplants 26–45 per cent and in the normal controls 135–200 per cent

After 11 weeks without treatment the mammary gland response to testosterone stimulation was studied As in previous studies (*Ahren* 1962), testosterone produced only a few alveoli in the rats with one

* Fellow of the Consejo Nacional de Investigaciones (Argentina)

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Andersen, Carl E (Institute of Biochemistry University of Aarhus Denmark) KINETIC STUDIES ON THE ELIMINATION OF ISONICOTINIC ACID HYDRAZIDE

The elimination of isonicotinic acid hydrazide (INH) was investigated after intravenous injection in 20 subjects The method of *Maher et al* (1957) was used to determine free and acetylated INH in plasma and urine

The rate of elimination was proportional to plasma concentration In one group of subjects the average halflife of INH was 55±6 min in another group 152±20 min in accordance with the results of *Jenne et al* (1961)

Determination of the drug alone shows elimination as a whole the determination of the metabolites allow a differentiation between some of the routes of elimination viz excretion through the kidneys acetylation and other forms of elimination. Assuming that elimination follows first order kinetics different elimination constants (k) may be calculated k_e (excretion calculated from renal clearance and distribution volume) and k_x (other forms of elimination) show a normal distribution among the 20 subjects and only k_a (acetylation) has a bimodal pattern 12 of the subjects having an average k_a of 0 01013±0 00170 and 8 subjects 0 00186±0 00025 Thus one group acetylates the drug more than 5 times as fast as the other group Based on the k values the participation of the different types of elimination in total elimination was calculated and compared with the findings in 24 h urine there was relatively good agreement between calculated and experimental values

Maher J R et al Amer Rev Tuberc 1957 76 852
Jenne J W et al Amer Rev resp Dis 1961 *84* 371

Abstr XI Scand Physiol Congr Copenhagen 1963
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Anden, N-E and T Magnusson (Department of Pharmacology, University of Goteborg Sweden) EFFECT OF NORADRENALINE DEPLETION BY ALPHA METHYL META TYROSINE, METARAMINOL AND d ADRENALINE

Reserpine blocks the effects of adrenergic, sympathetic nerve stimulation and decreases the content of noradrenaline in tissues With metaraminol or d adrenaline the noradrenaline content of peripheral tissues could be reduced by more than 95 per cent The injected amines were recovered in tissues in a molar concentration of 50 to 100 per cent of the missing noradrenaline As sympathomimetic agents they are more than ten times weaker than 1 noradrenaline Reduction in noradrenaline could also be produced by α-methyl meta tyrosine which is partly converted to metaraminol *in vivo* After the reduction of noradrenaline by these agents the following observations were made in rats and cats which had undergone unilateral cervical sympathectomy

1) There was no ptosis (rats) nictitating membrane relaxation (cats) or miosis (cats) on the intact side, while these signs were observed on the denervated side

2) The nictitating membrane was contracted (cats) the pupil dilated (cats and rats) and the eye protruded (cats and rats) during stimulation (< 10/sec) of the cervical sympathetic trunk The responses did not deviate significantly from those observed in untreated animals

3) The blood pressure responses in cats after bilateral carotid occlusion tyramine (0 5 mg/kg) and atropine plus carbacholine (0 2 mg/kg) were within the normal range

Thus displacement of more than 95 per cent of tissue noradrenaline by these less active analogues did not cause any detectable disturbance of adrenergic nerve functions

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Andersen, Carl E (Institute of Biochemistry University of Aarhus Denmark) KINETIC STUDIES ON THE ELIMINATION OF ISONICOTINIC ACID HYDRAZIDE

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Determination of the drug alone shows elimination as a whole the determination of the metabolites allow a differentiation between some of the routes of elimination viz excretion through the kidneys acetylation and other forms of elimination Assuming that elimination follows first order kinetics different elimination constants (k) may be calculated k_e (excretion calculated from renal clearance and distribution volume) and k_x (other forms of elimination) show a normal distribution among the 20 subjects and only k_a (acetylation) has a bimodal pattern 12 of the subjects having an average k_a of 0 01013±0 00170 and 8 subjects 0 00186±0 00025 Thus one group acetylates the drug more than 5 times as fast as the other group Based on the k values the participation of the different types of elimination in total elimination was calculated and compared with the findings in 24 h urine there was relatively good agreement between calculated and experimental values

Maher J R et al Amer Rev Tuberc 1957 *76* 852
Jenne J W et al Amer Rev resp Dis 1961 *84* 371

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Andersen, Harald T (Institute of Zoophysiology, University of Oslo, Norway, and Department of Physiology Kungl Veterinarhog skolan Stockholm Sweden) THE REFLEX NATURE OF THE PHYSIOLOGICAL ADJUSTMENTS TO DIVING AND THEIR AFFERENT PATHWAY

The elicitation of submersion apnoea and bradycardia was investigated in intact and decerebrated ducks with and without various trigeminal lesions, to study the reflex nature of the diving characteristics, and to explore their afferent pathway The circulatory as well as the respiratory adjustments to water immersion of the head are medullary reflexes independent of higher levels of the central nervous system, although the basic reflex may be influenced by activity in higher centers

The trigeminal nerve constitutes the afferent pathway of the reflex arc by which the reactions to diving are induced upon submersion Its ophthalmic division is the most important branch in this respect but the mandibular portion is an element in the afferent limb of the reflex arc as well The maxillary nerve does not serve such function

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Andersson, Sven A and Ulf Norrsell (Institute of Physiology University of Goteborg Sweden) THE LOCALIZED ALPHA ACTIVITY IN THE SOMATOSENSORY CORTEX AND ITS ACTIVATION VIA AN ASCENDING VENTRAL SPINAL PATHWAY

In the spinal cord of the cat the specific projection system consists of the dorsal columns and a pathway ascending in the dorsomedial part of the lateral funiculus An interruption of this projection system caused the appearance of regular high voltage slow waves in the somatosensory cortex (*Andersson* 1962 a) The spontaneous frequency of these slow waves was 8–12/sec *i e* within the limits of the cortical alpha rhythm This slow wave activity was strictly localized to the parts of the somatosensory areas that correspond to peripheral fields situated below the level of the lesion A continuity in the slow waves could be observed only when recording below the cortical surface with maximum amplitude at a depth of 800–1000 μ In surface recordings the slow wave activity appeared as an increased frequency of spindle bursts

Strong stimuli applied to peripheral fields anywhere below the level of the spinal lesion induced an increase in both the amplitude and frequency of the slow waves (*Andersson* 1962 b) This effect depended on a suitable depth of anaesthesia A stimulus which cause this effect on the slow waves suppressed spindle bursts and induced a low voltage high frequency activity in other cortical areas The bilateral ventral flexor reflex tract mediates these effects (*Lundberg and Oscarsson* 1962)

Andersson S A Med exp (Basel) 1962 a *6* 21–24
Andersson S A Med exp (Basel) 1962 b *6* 25–28
Lundberg A and O Oscarsson Acta physiol scand 1962 *54* 270–286

Abstr XI Scand Physiol Congr Copenhagen 1963
Acta physiol scand 1963 *59* Suppl no *213*

Andersson, Sven A and Ulf Norrsell (Institute of Physiology, University of Goteborg, Sweden) THE SUPPRESSION OF ALPHA ACTIVITY IN A CORTICAL RECEIVING AREA BY ACTIVITY IN ITS SPECIFIC PROJECTON PATHWAYS

Interruption of the specific projection pathways in the spinal cord caused the appearance of regular slow waves within the frequency limits of the alpha rhythm in the somatosensory cortex (*Andersson* 1962) This slow wave activity could be obtained also by partial lesions of these pathways In preparations with such lesions microelectrode recordings indicated that different effects on the slow waves could be induced from different peripheral fields situated below the level of the lesion Thus, in the presence of cells that can be activated with short latency by light stimuli applied in small contralateral fields, the slow waves were abolished when these cells were activated However, strong stimuli, applied in peripheral fields from which no such cell activation could be elicited induced an increase in the amplitude and frequency of the slow waves

The present observations suggest that the activity in the specific projection pathways prevents the appearance of slow waves in their cortical receiving areas in the intact animal

Andersson S A Med exp (Basel) 1962 *6* 21-25

Abstr XI Scand Physiol Congr Copenhagen 1963
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Andersen, Svend Olav and Berit Kristensen (Zoophysiological Laboratory B University of Copenhagen, Denmark) INCORPORATION OF PHENYLALANINE AND TYROSINE IN THE CROSS-LINKAGES OF A PROTEIN RESILIN

The rubber like protein resilin present in some parts of the cuticle of insects contains some very stable cross linkages between the peptide chains (*Weis Fogh* 1960) Two fluorescent compounds have been isolated from hydrolysates of locust resilin and although their chemical structure is still unknown it has been shown that one of them is a diaminodicarboxylic acid and the other a triaminotricarboxylic acid (*Andersen* 1963) There is evidence that these compounds constitute the cross linkages in resilin

As both compounds are aromatic and contain at least one phenolic group it was thought probable that phenylalanine and/or tyrosine could act as precursors in the animals Experiments were performed where locusts (*Schistocerca gregaria*) were injected one day after emergence with tyrosine or phenylalanine generally marked with C^{14} When the animals were given radioactive phenylalanine the activity incorporated in resilin could afterwards be found distributed between phenylalanine tyrosine and the two fluorescent compounds When radioactive tyrosine was used the activity was restricted to tyrosine and the two compounds but nothing was present in phenylalanine

This indicates the following sequence in the synthesis of the cross linkag s in resilin

phenylalanine → tyrosine → cross linkages

Th~ specific activities of the isolated compounds were so much higher than that of the isolated tyrosine that it can be assumed that more than one tyrosine residue is involved in the biosynthesis of one cross linkage

The incorporation of radioactive tyrosine into resilin has also been studied by means of autoradiography which shows that the incorporation is very rapid

Andersen S O Biochim biophys Acta 1963 69 249–262
Weis Fogh T J exp Biol 1960 37 889–907

Abstr XI Scand Physiol Congr Copenhagen 1963
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Appelberg, B and I Z Kosary (Department of Physiology, Kungl Veterinärhogskolan, Stockholm, Sverige) RECIPROCAL FUSIMOTOR INFLUENCE FROM THE RED NUCLEUS

The experiments were performed on cats lightly anaesthetized with Nembutal® The effect was studied of electrical stimulation in the region of the red nucleus upon the activity in contralateral fusimotor efferent fibres and in muscle spindle afferents from the gastrocnemius (extensor) and tibialis anterior (flexor) muscles The activity in most fusimotor fibres was inhibited from a region comprising the dorsal part of and extending slightly dorsally to the red nucleus From the same region the response to static stretch was diminished in extensor as well as flexor muscle spindle afferents From the ventral part of the red nucleus the activity in many fusimotor fibres was facilitated and the static response of flexor spindles was increased Extensor spindle discharge was inhibited also from this region The reciprocal effect on muscle spindles from the ventral part of the red nucleus was often accompanied by a contraction in the flexor muscle under study The elicitation of the contraction was independent of whether the ipsilateral dorsal roots were intact or not, and thus due to concomitant activation of alpha motoneurones to flexor muscles *Sasaki Namikawa and Hashiramoto* (1960) have shown that rubral stimulation influences alpha motoneurones in a reciprocal way *i e* facilitation of flexor and inhibition of extensor neurones Fusimotor neurones to flexor and extensor muscle spindles are controlled in a similar manner The area the stimulation of which yielded the reciprocal effect was the same as the part of the red nucleus previously shown to have anatomical connections with the lumbar spinal cord (*Pompeiano and Brodal* 1957) The dorsal part of the nucleus which according to these authors, mainly has fore limb connections exerted an inhibitory influence on both flexor and extensor spindles in hind limb muscles indicating some sort of reciprocity of rubral effects on fore and hind limbs

Pompeiano O and A Brodal Experimental demonstration of a somatotopical origin of rubrospinal fibres in the cat *J comp Neur* 1957 *108* 225–252

Sasaki K A Namikawa and S Hashiramoto The effect of midbrain stimulation upon alpha motoneurones in lumbar spinal cord Jap J Physiol 1960 *10* 303–316

Abstr XI Scand Physiol Congr Copenhagen 1963
Acta physiol scand 1963 59 Suppl no 213

Areskog N H , G Arturson and G Grotte (Departments of Clinical Physiology Physiology and Surgery University of Uppsala Sweden) STUDIES ON HEART LYMPH KINETICS OF ^{131}I ALBUMIN IN THE DOG HEART LUNG PREPARATION

The transport of ^{131}I albumin from plasma to lymph was studied in dog heart lung preparations Cannulation of a lymph vessel mainly draining the left ventricle was performed After injection of the labelled substance plasma mixing was complete within a few minutes The plasma concentration of the tracer was steady for two to three hours During this time plasma and heart lymph samples were collected The concentration increase of the labelled substance in heart lymph showed a time course indicating that the substance from the *intra* vascular compartment was mainly distributed to a single *extra* vascular compartment The data could thus be fully explained by a two compartment model and the permeability constants as well as the approximate size of the extravascular albumin space (drained by the lymphatic cannula) could be calculated

In one out of seven successful experiments the data were as follows heart weight 88 g total extravascular albumin space 11 9 ml extravascular albumin space drained by the lymphatic cannula 6 1 ml amount of albumin from the *intravascular* compartment filtered across the cardiac capillaries (rate constant) 0 00014 per min amount of albumin from the *extravascular* compartment transported away by the lymphatics (rate constant) 0 0067 per min turnover time or average time of albumin molecules in the extravascular space 150 min

The present experimental approach therefore allowed *quantitative* determination of certain aspects of lymph flow and compartmental distribution of the cardiac extravascular space drained by the cannulated lymph vessel The extravascular fluid and the lymph of the dog s heart may be regarded as identical fluids as reflected by the albumin concentration.

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Arstila, M , L Hirvonen and T Peltonen (Department of Physiology University of Turku Finland) DUCTUS VENOSUS AND THE VEGETATIVE NERVES

The *ductus venosus* in the lamb remains open for 3 to 4 days after birth According to *Barron* (1942) there is a sphincter in the ductus which is innervated by the vagus The flow of radiopaque injected into the portal sinus through the umbilical vein was recorded with cineradiography in newborn lambs anesthetized with chloral ose Aortic and right ventricular or atrial pressures were recorded simultaneously During unilateral vagal stimulation the *ductus venosus* opened or widened in 9 of 12 lambs In three there were no es sential changes in the calibre of the ductus The calibre of the ductus varied during sympathetic stimulation elicited either by bilateral carotid clamping or by intravenous injection of epinephrine Ace tylcholine and norepinephrine never closed the ductus when ad ministered intravenously, after intraumbilical injection the calibre of the ductus increased

Barron Anat Rec 1942 *82* 398

Abstr XI Scand Physiol Congr Copenhagen 1963
Acta physiol scand 1963 59 Suppl no 213

Asmussen Erling, S H Johansen, Mogens Jørgensen and Marius Nielsen (Laboratory for the Theory of Gymnastics Zoophysiological Laboratory A University of Copenhagen and Gentofte County Hospital Denmark) THE NEUROGENIC FACTORS IN THE REGULATION OF RESPIRATION AND CIRCULATION DURING MUSCULAR EXERCISE

It has been demonstrated that neurogenic factors are of importance for the regulation of respiration and circulation during muscular exercise We have performed a series of experiments in which these nervous influences have been varied in two different ways while maintaining a constant rate of work 1) Blocking the circulation to the working muscles and 2) giving small doses of curare to the exercising subject *In the former* case the ensuing *anaerobiosis* in the blocked muscles will diminish the work output of every single active muscle fiber thus necessitating the recruitement of an increasing number of active muscle fibers for the maintenance of the work In the second case the curare will block the transmission at a certain number of motor endplates and thus necessitate the spreading of the excitation to a larger number of motor units In both cases pulmonary ventilation increased considerably when the alveolar P_{CO_2} was maintained constant by adding CO_2 to the inspired air Pulse rate and arterial blood pressure behaved differently in the two cases With blocked circulation both increased considerably whereas in the curare experiments they only showed small changes

In both series of experiments it can be excluded that the observed increase in respiratory and circulatory functions were due to changes in the composition of the circulating arterial blood But the observed effects might be interpreted by the action or interaction of nervous factors viz. 1) afferent impulses from intramuscular chemoreceptors 2) mechano-receptors or 3) spread of excitation from the higher motor centers to the regulatory centers

As the increase in respiration in the curare experiments where no local anaerobiosis was present was of the same magnitude as in the blocking experiments it seems justifiable to assume that the observed respiratory changes were not due to a stimulation of local chemoreceptors

Since pulse rate and blood pre sure were considerably more in

creased in the blocking experiments than in the curare experiments it can not be excluded that intramuscular chemoreceptors take part in the regulation of these functions Work experiments with artificial stimulation of the muscles in humans or animals have shown that the higher motor centers are not of primary importance for the adaptation of respiration and circulation to work In spite of the fact that the rate of work was maintained constant in the present series of experiments an increased activity in the mechanoreceptors can be assumed if the gamma loop is involved in this kind of exercise

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Acta physiol scand 1963 59 Suppl no *213*

Bergstrom R M , Lea Bergstrom and P Putkonen (Institute of Physiology University of Helsinki Finland) THE EFFECT OF THALIDOMIDE ON THE ELECTROENCEPHALOGRAM (EEG) OF THE INTRAUTERINE GUINEA PIG FOETUS

Brain waves were recorded in intrauterine guinea pig foetuses in acute and chronic preparations (*Bergstrom* 1962) during the administration of thalidomide (α N phthalimidoglutarimid perorally up to 300 mg/kg to the pregnant animal intracardially in propyleneglycol up to 4 mg/kg to the foetus or the pregnant animal) The EEG and the electrocorticogram of the pregnant animal and of the foetus were recorded simultaneously Doses of thalidomide (100 mg/kg perorally or 2–3 mg/kg intracardially) which just increased the alpha activity in the electrocorticogram of the pregnant animal depressed the foetal EEG most pronounced in young foetuses

Bergstrom R M Prenatal development of motor functions Ann Chir Gynaec Fenn 1962 *51* Suppl *112* 1–48

Abstr XI Scand Physiol Congr Copenhagen 1963
Acta physiol scand 1963 *59* Suppl no *213*

Bernhard, Carl Gustaf (Department of Physiology II, Karolinska Institutet, Stockholm, and the Rockefeller Institute, New York)
ON THE FUNCTIONAL SIGNIFICANCE OF THE PIGMENT MIGRATION IN THE COMPOUND EYE

The present study represents a continuation of the comparative electrophysiological and histological investigations on the functional significance of the positional changes of the secondary iris pigment in the compound eye of superposition type *(Bernhard and Ottoson* 1959, 1960, 1961, 1962) Local illumination of a night moth s (*Prodenia eridiana*) dark-adapted eye is followed by a migration of the secondary iris pigment from dark to light position which is restricted to the ommatidia illuminated and to the surrounding ommatidia The lack of contralateral effects as well as the spatial limitation of the effect of light on the eye illuminated show the local character of the pigment migration induced by light in nocturnal Lepidoptera

Bernhard C G and D Ottoson Acta physiol scand 1959 *47* 383
Bernhard C G and D Ottoson J gen Physiol 1960 *44* 205
Bernhard C G and D Ottoson Acta physiol scand 1961 *52* 99
Bernhard C G and D Ottoson Acta physiol scand 1962 *54* 95

Abstr XI Scand Physiol Congr Copenhagen 1963
Acta physiol scand 1963 59 Suppl no *213*

Brummerstedt Hansen, E (Department of Physiology Endocrinology and Bloodgrouping The Royal Veterinary and Agricultural College Copenhagen) IMMUNOELECTROPHORETIC IN VESTIGATIONS ON SERUM PROTEINS IN PIG FE TUSES

Fetal pig serum was studied by means of immunoelectrophoresis using microimmunoelectrophoresis (*Scheidegger* 1955) with veronal buffer pH 8 6 containing calcium lactate (*Hirschfeld 1960*) and an electric field of 5 V/cm for 90 minutes The antibody was serum from rabbits immunized with serum from adult pigs The youngest fetuses from which sufficient blood could be obtained were 25 days old and weighed on the average 0 5 g. At this age there were three distinct arcs designated 1 2 and 3, with the same mobility as al bumin α and β globulins in adult serum In addition there were two or three smaller arcs one of them placed laterally to arc no 1 stained with Sudan black From the youngest age group to the next (35 days old) the number of arcs increased by four or five smaller arcs The number of arcs at the age of 40 days was less than at 35 days possibly due to individual variations from one litter to an other In the next six age groups up to 112 days old the varia tions were small from one group to the following New small arcs appeared during fetal life and at 112 days there were about fifteen as compared with about 20 in adult pigs As expected the most essential difference was the absence of γ globulin in the fetal serum To determine to which components in the adult serum arcs no 1, 2 and 3 were related combined diffusion experiments (*Clausen and Heremans* 1960) were made Arc no 3 corresponded to a distinct arc in the β field of the adult serum, a small arc in the curvature of no 1 was immunologically identical with albumin while no 1 itself was related to a small arc on the anodic part of the albumin arc Some of the arcs in the curvature of no 2 corresponded to arcs in the α field whereas no 2 itself could not be identified

Scheidegger J J Int. Arch Allergy 1955 7 103–110
Hirschfeld J Science Tools 1960 7 18–25
Clausen J and J Heremans J Immunol 1960 84 128–134

Abstr XI Scand Physiol Congr Copenhagen 1963
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Bygdeman, S (Department of Physiology Karolinska Institutet, Stockholm, Sweden) REACTIVITY OF CAT SKELETAL MUSCLE VESSELS TO NORADRENALINE DURING INDUCED CHANGES IN ACID BASE BALANCE OF THE BLOOD

The effect of noradrenaline administration on peripheral vascular resistance has been followed both before and during experimentally induced changes in the acid base balance of the blood The vascular resistance in skeletal muscles was studied in a skinned hind leg perfused with the cat s own blood, employing a constant outflow pump Systemic blood pressure, perfusion pressure, arterial blood pH and CO_2 tension in end tidal air were recorded continuously

The increase in perfusion pressure, normally induced by an intra arterial injection of noradrenaline, was moderately diminished 17 per cent or 40 per cent, by ventilation with 10 or 20 per cent CO_2 in O_2 To maintain a constant response it was found necessary to increase the noradrenaline dose by a factor of either 1 9 or 3 3 After sympathetic denervation the values were 11 or 39 per cent and 1 4 or 1 9

During respiratory alkalosis the peripheral noradrenaline response increased by 14 per cent at pH 7 56 and 32 per cent at pH 7 80, in the denervated, skinned leg The dose necessary to elicit an unchanged response could at the same time, be decreased to 0 71 and 0 45 of the control dose The mean CO_2 tension in end tidal air decreased from 40 to 19 and 13 mm Hg respectively A corresponding fall in CO_2 tension at a constant pH on the other hand did not cause the same increase in peripheral response suggesting that this increase was primarily due to the pH change and not to the concomitant decrease in CO_2 tension

It has previously been shown (*Bygdeman and Euler* 1962) that acidosis also diminishes the noradrenaline effect in an unskinned leg Moreover, the effect of the acidosis is more pronounced in this preparation than in the skinned leg This difference can be explained by a redistribution of blood from areas of less reduced reactivity (muscular vessels) to those of more reduced reactivity (skin vessels) during respiratory acidosis

Bygdeman S and U S v Euler Acta physiol scand *1962* *54* 138–146

Abstr XI Scand Physiol Congr Copenhagen 1963
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Carlsen F, F Fuchs* and G G Knappeis (Laboratory of Biophysics and Institute of Neurophysiology University of Copenhagen Denmark) SARCOMERE LENGTH AND CONTRACTILITY IN GLYCEROL EXTRACTED MUSCLE FIBRES

According to the sliding filament model of contraction (*H E Huxley* and *Hanson* 1954 and *A F Huxley* and *Niedergerke* 1954) the ability of vertebrate skeletal muscle to generate tension and shorten should fail at a sarcomere length of ~ 3 5 μ, this being the length at which there is no longer any overlap of A and I filaments Attempts to confirm this prediction using living isolated frog fibres have led to conflicting conclusions (*Carlsen et al* 1961 *A F Huxley* and *Peachey* 1961) Interpretation of these experiments is complicated by the fact that distensibility of the living fibre is greater in the middle than at the tendon ends this difference being reflected in a corresponding inhomogeneity in sarcomere length Also the elastic recoil of the stretched living fibre makes it impossible to study the behavior of the fibre with forces less than the applied stretching force Both of these difficulties can be avoided by the use of glycerol extracted fibres A preliminary attempt in this direction using thin fibre bundles has been reported by *Edman* (1962) The results reported here are based on measurements of isotonic shortening as a function of sarcomere length in lightly loaded (100–200 g/cm) single glycerol extracted rabbit psoas fibres

Psoas bundles stretched in the living state were extracted with a 50 per cent glycerol water mixture containing 1 mM *phosphate* pH 7 0 A single fibre of about 50 μ diameter with a platinum weight attached to one end was suspended freely in a buffer solution contained in a glass cuvette mounted on the stage of a horizontal measuring microscope In each experiment the sarcomere length and fibre diameter were measured at successive points along the fibre by means of an ocular micrometer with movable cobweb Following the measurement of fibre length adenosine triphosphate (ATP 5 mM) was added and the new *length determined* at measured time intervals The magnitude and homogeneous distribution of sar

* Post-doctoral Fellow of the U S Public Health Service

comere lengths in both transverse and longitudinal directions has been verified by light and electron microscopy of fibres from the same bundles as *the fibres used in the isotonic experiments* as well as by photometric tracings of microphotographs

Experiments have been performed with fibres of initial sarcomere length of 2 6–4 5 μ That the overlap of filaments disappears at a sarcomere length of 3 4–3 5 μ has been confirmed by electron microscopy The results show that for the range of loads employed the degree of shortening was essentially invariant (75–85 per cent) *up to a sarcomere length of* ~ *4 2 μ Beyond this length there* was great variability (shortening 15–85 per cent)

Electron micrographs of highly stretched fibres made before and after ATP induced shortening indicate that shortening in this situation occurs through a curling or disorganization of the I filaments, no evidence being found for a movement of I filaments into the A band These findings would suggest that as far as glycerol *extracted muscle is concerned, the fundamental contractile event is* some structural reorganization within the I filaments

Carlsen F G G Knappeis and F Buchthal J biophys biochem Cytol *1961 11 95*

Edman K A P Conference on the Chemistry of Muscle Contraction *Dedham Mass 1962*

Huxley A F and R Niedergerke Nature (Lond) *1954 173 971*

Huxley A F and L D Peachey J Physiol (Lond) 1961 *156* 150

Huxley *H* E and J Hanson Nature (Lond) *1954 173 973*

Abstr XI Scand Physiol Congr Copenhagen 1963
Acta physiol scand 1963 59 Suppl no 213

Carlsen, F and G G Knappeis (Laboratory of Biophysics and Institute of Neurophysiology University of Copenhagen Denmark) FURTHER INVESTIGATIONS OF THE ULTRASTRUCTURE OF THE Z DISC IN SKELETAL MUSCLE

In a recent paper (*Knappeis* and *Carlsen* 1962) we have put forward a model of the Z disc in cross striated muscle The model is based on observations in the electron microscope of both longitudinal and cross sections of frog muscle The Z disc consists of filaments forming pyramids the I filaments being attached to the peaks of the pyramids In longitudinal sections an I filament on one side of the Z disc is situated in between two I filaments on the opposite side In cross sections through the Z region the I filaments are arranged in a tetragonal pattern in cross sections through the zone of overlap they are arranged in a hexagonal pattern We have calculated the displacement of the I filaments during their course from the zone of overlap to the Z disc which would account for the transition from a hexagonal to a tetragonal pattern

Based on the regularity with which the filaments are arranged elsewhere in the fibre we have assumed that the tetragons are regular squares A displacement of each I filament of 90 A along their course would account for a change of the square arrangement to a hexagonal one However the hexagons obtained in this way were not exactly regular

An analysis in cross sections of the Z disc where the cutting direction could be traced by knife marks and where these marks were perpendicular to one of the arrays in the tetragonal lattice revealed that the lattice in fact is not square but rhombic with angles deviating from orthogonality by 8 to 9 degrees A rearrangement of the I filaments from a rhombic lattice to a regular hexagonal lattice by a displacement of the I filaments requires that the rhombic lattice deviates from orthogonality by an angle of 8 2 degrees

Knappeis G G and F Carlsen J Cell Biol 1962 *13* 323

comere lengths in both transverse and longitudinal directions has been verified by light and electron microscopy of fibres from the same bundles as the fibres used in the isotonic experiments as well as by photometric tracings of microphotographs

Experiments have been performed with fibres of initial sar comere length of 2 6–4 5 μ That the overlap of filaments disappears *at a sarcomere length of 3 4–3 5 μ has been confirmed by electron* microscopy The results show that for the range of loads employed the degree of shortening was essentially invariant (75–85 per cent) *up to a sarcomere length of ~ 4 2 μ Beyond this length there was* great variability (shortening 15–85 per cent)

Electron micrographs of highly stretched fibres made before and after ATP-induced shortening indicate that shortening in this situation occurs through a curling or disorganization of the I fila ments, no evidence being found for a movement of I filaments into the A band These findings would suggest that as far as glycerol extracted muscle is concerned, the fundamental contractile event is some structural reorganization within the I filaments

Carlsen F G G Knappeis and F Buchthal J biophys biochem Cytol 1961 *11* 95
Edman K A P Conference on the Chemistry of Muscle Contraction Dedham Mass 1962
Huxley A F and R Niedergerke Nature (Lond) 1954 173 971
Huxley A F and L D Peachey J Physiol (Lond) 1961 *156* 150
Huxley H E and J Hanson Nature (Lond) 1954 *173* 973

Abstr XI Scand Physiol Congr Copenhagen 1963
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Crone, Christian (Physiological Laboratory University of Cambridge England) * WHICH PART OF THE CENTRAL NERVOUS SYSTEM ACTIVATES THE ADRENAL MEDULLA DURING HYPOGLYCEMIA?

Transection of the splanchnic nerves abolishes the adrenal response to stimuli such as hypoglycemia or anoxia An increased output of catecholamines therefore reflects activity in the central part of the sympathetic system I have investigated whether certain parts of the central nervous system elicit the adrenal response by comparing the release of catecholamines in intact decerebrate and spinal animals (Welsh Mountain and Border Leicester sheep anesthetized with either Nembutal® or chloralose)

Decerebration was performed at the superior collicular level transection of the spinal cord immediately beneath the foramen magnum Effluent blood was collected from the left adrenal gland Hypoglycemia was induced by intravenous injection of insulin Catecholamines were determined by fluorimetry on plasma eluates

In *intact* animals an increased output of adrenaline occurred within 30–60 min after injection of insulin A definite but much smaller increase in the rate of secretion of noradrenaline was observed in many experiments notably when chloralose was used *Decerebration* in itself elicits a secretion of catecholamines, but a supervening hypoglycemia amplified the response which then became of the same order of magnitude as in intact animals The output of adrenaline and noradrenaline in hypoglycemic *spinal* sheep was not significantly different from the resting secretion in intact animals with normal blood sugar

The experiments show that the parts of the central nervous system which remain after decerebration can activate the adrenal medulla in hypoglycemia The region responsible for this activation is some part of the brain stem as the isolated spinal cord is incapable of activating the adrenal medulla in hypoglycemia Whether the hypothalamus plays any role in the activation pattern in intact animals cannot be deduced from these experiments

* Present address Institute of Medical Physiology University of Copenhagen Denmark The work was carried out on leave of absence from the University of Copenhagen

Abstr XI Scand Physiol Congr Copenhagen 1963
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Christensen, S (Department of Physiology, University of Aarhus Denmark) TRANSFER OF LABELLED CHOLESTEROL FROM PLASMA TO AORTIC INTIMA-MEDIA

Controls and cholesterol fed cockerels were given an intravenous injection of 8 5 ml of blood which had previously been incubated for two hours at 42°C with 10μC of 4–C^{14} cholesterol The birds received their own blood

The radioactivity per cm² of an aortic intima media layer after 1 to 24 hours was expressed as a function of time and of average lipid C^{14} concentration in the plasma during the experiment The resulting expression has the dimension volume/area/time The *average value from experiments with 25 cholesterol fed birds* was 0 046±0 005 μl/cm²/h When divided into groups according to experimental time (1–2, 4, 5 and 24 hours) no significant differences were found The average value for five control birds was 0 09±0 02 μl/cm²/h in experiments lasting four hours

The rates of transfer of plasma cholesterol across the intimal surface were calculated for the individual birds as the product of the plasma cholesterol concentration (μg/μl) and the intimal clearance ' (μl/cm /h), the latter being taken as a measure of the plasma volume containing as much cholesterol as was transferred from the plasma across one cm of *intimal surface in one hour The transfer* rates thus obtained showed a significant, positive co-variation with the cholesterol concentrations of the various aortic intima media layers ($P < 0\ 001$, n = 25)

The transfer of labelled cholesterol is believed to depend mainly upon a transfer of whole plasma lipoprotein molecules (*Christensen* 1961, 1962)

Christensen S J Atheroscler Res 1961 *1* 140
Christensen S J Atheroscler Res 1962 *2* 131

Abstr XI Scand Physiol Congr Copenhagen 1963
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Abstr XI Scand Physiol Congr Copenhagen 1963
Acta physiol scand 1963 *59* Suppl no *213*

Cuddy, T, J Stenberg, P-O Astrand and B Saltin (Department of Physiology Gymnastiska Centralinstitutet, Stockholm Sweden) CARDIAC OUTPUT DURING SUBMAXIMAL AND MAXIMAL EXERCISE

In the present study oxygen uptake, oxygen content of arterial blood, cardiac output and stroke volume were determined by the dye dilution technique of Steward Hamilton in 11 women and 12 men, 20 to 31 years of age, at rest and during submaximal and maximal work At rest, plasma volume (T-1824) and heart volume were determined Sitting on the bicycle ergometer the stroke volume was 40 to 90 per cent of the maximum value attained during exercise Maximal stroke volume was reached at a work load with an oxygen uptake of about 40 per cent of the maximum and a heart rate of about 110 No tendency to a decrease in stroke volume was noticed with maximal work The variation in stroke volume was ±4 per cent during exercise in the range from 40 to 100 per cent of aerobic capacity The correlation between heart and stroke volume on the one hand and cardiac output on the other was high and as expected from the dimensions of the individual With submaximal and maximal exercise women had a higher cardiac output per liter oxygen uptake than men, explicable by the lower concentration of hemoglobin in female blood

Astrand P O and B Saltin Oxygen uptake during the first minutes of heavy muscular exercise J appl Physiol 1961 *16* 971
Dow P Estimation of cardiac output and central blood volume by dye dilution Physiol Rev 1956 *36* 77
Asmussen E and M Nielsen The cardiac output in rest and work Acta physiol scand 1953 *27* 217
Asmussen E and M Nielsen The cardiac output in rest and work at low and high oxygen pressures Acta physiol scand 1955 *35* 73
Grimby G and N J Nilsson Cardiac output during exercise pyrogen induced fever Scand J clin Lab Invest *15* (suppl 69)
Wang Y R J Marshall and J T Shephard The effect of changes in posture and of graded exercise on stroke volume in man J clin Invest 1960 *39* 1051
Wang Y J T Shephard R J Marshall L Rowell and H L Taylor Cardiac response to exercise in unconditioned young men and in athletes Circulation 1961 *24* 1064
Jonsell S Determination of the heart size Acta radiol (Stockh) 1939 *20* 325

Abstr XI Scand Physiol Congr Copenhagen 1963
Acta physiol scand 1963 *59* Suppl no *213*

Doving Kjell B and Gosta Gemne (Department of Physiology II Karolinska Institutet Stockholm Sweden) FIBER SPECTRA AND COMPOUND ACTION POTENTIAL OF THE OLFACTORY TRACT IN FISH (*Lota lota* L)

The olfactory tract of the burbot has been studied by histological and electrophysiological methods Electrical stimulation of the olfactory nerve and the contralateral tract showed that the major part of the tract is made up by afferent fibers Evidence was also obtained for the presence of efferent fibers This confirms earlier histological findings of efferent fibers in the olfactory tract of bony fishes (*Sheldon* 1912 *Holmgren* 1920)

Before entering the telencephalon the tract splits into a lateral and a medial fiber bundle each of which consists of two portions Histological examinations showed that the two main fiber bundles contained myelinated nerve fibers from about 1 μ to 6 μ in diameter The majority of the fibers in the lateral portion of the medial bundle were smaller than 1 μ

The compound action potential of the olfactory tract had three peaks with conduction velocities of 2 7 1 5 and 0 14 m/sec at 10° C The action potential exhibited the first two peaks in each of the four different portions while the third elevation was present only in the lateral portion of the medial bundle

The absolute refractory periods of the three elevations of the compound action potentials were about 3 5 8 and 15 msec The after potentials were studied by trains of electric shocks Repetitive stimulation of the bundles yielding the first two elevations gave prolonged positive after potentials after cessation of stimulation The third component of the compound action potential was not followed by a positive after potential but had a negative after potential which increased in amplitude upon tetanization

Sheldon R E The olfactory tracts and centers in teleosts J comp Neurol 1912 *22* 177–340
Holmgren N Zur Anatomie und Histologie des Vorder und Zwischen hirns der Knochenfische Acta zool 1920 *1* 137–315

Abstr XI Scand Physiol Congr Copenhagen 1963
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Cuddy, T , J Stenberg, P O Åstrand and B Saltin (Department of Physiology, Gymnastiska Centralinstitutet, Stockholm Sweden) CARDIAC OUTPUT DURING SUBMAXIMAL AND MAXIMAL EXERCISE

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Dow P Estimation of cardiac output and central blood volume by dye dilution Physiol Rev 1956 *36* 77
Asmussen E and M Nielsen The cardiac output in rest and work Acta physiol scand 1953 *27* 217
Asmussen E and M *Nielsen The cardiac output in rest* and work at low and high oxygen pressures Acta physiol scand *1955 35 73*
Grimby G and N J Nilsson Cardiac output during exercise pyrogen induced fever Scand J clin Lab Invest *15* (suppl 69)
Wang Y R J Marshall and J T Shephard The effect of changes in posture and of graded exercise on stroke volume in man J clin Invest 1960 *39* 1051
Wang Y J T Shephard R J Marshall *L Rowell and H L Taylor* Cardiac response to exercise in unconditioned young men and in athletes Circulation 1961 *24* 1064
Jonsell S Determination of the heart size Acta radiol (Stockh) 1939 *20* 325

Abstr XI Scand Physiol Congr Copenhagen 1963
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Eisenman, J , G From, S Landgren and D Novin (Institute of Physiology University of Goteborg Sweden) THE ASCENDING PROJECTIONS OF CELLS IN THE MAIN SENSORY TRIGEMINAL NUCLEUS AND IN THE ROSTRAL SUBDIVISION OF THE NUCLEUS OF THE SPINAL TRIGEMINAL TRACT IN THE CAT

The ascending projections of cells in the caudal part of the nucleus of the spinal trigeminal tract in the cat were investigated by *Gordon Landgren* and *Seed* (1961) In the present series a similar investigation was carried out in the rostral subdivision of this nucleus and in the main sensory trigeminal nucleus of the cat anaesthetized with sodium pentobarbital The extracellular action potentials were obtained from single cells with a conventional microelectrode technique The location of their receptive fields and the types of effective physiological stimuli were recorded The region of the contralateral trigeminal lemniscus and the ipsilateral dorsal ascending trigeminal tract were stimulated electrically with a grid of tungsten electrodes at the mesencephalic level Responses with short and constant latency and with ability to follow stimulating frequencies of 1000/sec were considered antidromic The location of the stimulating electrodes and the recording position within the trigeminal nucleus were determined histologically

Trigeminal cells responding to light mechanical stimulation with in small receptive fields were localized to a dorsal and lateral shell within the nucleus There was a precise somatotopic organization within this part of the nucleus

A high incidence of upward projection (50–60 per cent) was found among the cells belonging to the group described above Cells with perioral and periorbital receptive fields always projected up the contralateral trigeminal lemniscus and those with oral receptive fields without exception projected into the ipsilateral ascending trigeminal tract.

An upward projection was less common (25 per cent) in another group of cells with large receptive fields and responding to nociceptive stimuli These cells were located medially and centrally in the trigeminal nucleus

There was no principal difference between the organization of

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Edman, K A P (Department of Pharmacology, Medical School Umea Sweden) ON THE ROLE OF CALCIUM IN THE EXCITATION CONTRACTION PROCESS OF SKELETAL MUSCLE

Removal of calcium from the extracellular fluid produces a gradual decline of the resting potentials of individual frog sartorius fibres, sufficiently deep to bring all fibres in the muscle into an inexcitable state The resting potential level at which excitability is lost (about 60 mV at 0–1° C) in the absence of calcium, is not significantly different from the level at which fibres become electrically inexcitable when calcium is present in the bath in a physiological concentration However, instead of an all or nothing failure as in the presence of calcium, there is a progressive decline of both the amplitude of the action potential and the size of the twitch response of individual fibres in parallel with the lowering of the resting potential in the calcium free Ringer s solution Calcium lack furthermore produces decremental propagation of the action potential as established by intracellular recording with a glass capillary electrode at various distances from the site of stimulation in frog sartorius fibres (*Edman* and *Grieve* 1963 a, 1963 b)

It is concluded that calcium is needed in the mechanisms governing the maintenance of the resting potential of the fibre membrane the production of the action potential and also the normal propagation of the action potential along the fibre The complete mechanical failure of individual fibres and the whole muscle in response to electrical stimulation in calcium free medium can be accounted for by inexcitability of the cell membrane The progressive reduction of the twitch output of individual fibres before they become inexcitable is probably, to a great extent, due to deficient activation of the contractile system by the affected action potential

Evidence in favour of a calcium dependent step in the contraction process of skeletal muscle beyond the excitation of the cell membrane has been obtained in the denervated rat diaphragm This preparation still responds with contracture to acetylcholine at a stage where the twitch response to electrical stimulation and the contracture response to complete depolarization with potassium have been lost

Edman K A P and D W Grieve Experientia 1963 a *19* 40

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Acta physiol scand 1963 59 Suppl no *213*

Ekblom, B., J Royce, B Saltin, J Stenberg and P O Åstrand
(Department of Physiology Gymnastiska Centralinstitutet Stockholm Sweden) CARDIAC OUTPUT DURING EXERCISE WITH SMALL AND LARGE MUSCLE GROUPS

Cardiac output (dye dilution technique) oxygen uptake heart rate blood lactates and arterial blood pressure were determined in subjects performing (i) arm work (cranking) (ii) leg work (cycling) and (iii) arm plus leg work (cranking + cycling) in (I) sitting and (II) supine position Submaximal as well as maximal work load were performed

the main sensory nucleus and the rostral subdivision of the nucleus of the spinal trigeminal tract The results suggest that the cells relaying precise tactile information to the thalamus form longitudinal columns extending along the dorsal and lateral border of the trigem inal nucleus of the cat

Gordon G S Landgren and W A Seed The functional characteristics of single cells in the caudal part of the spinal nucleus of the trigeminal nerve of the cat J Physiol (London) 1961 *158* 533–559

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Elwin C E and G Nilsson (Department of Pharmacology Karolinska Institutet Stockholm Sweden) COMPARISON OF THE EFFECT ON GASTRIC ACID SECRETION OF SOME PROTEIN COMPOUNDS RELEASING GASTRIN

Release of gastrin is supposed to occur when meat extracts polypeptides alcohols and other substances are in contact with the antrum mucosa The release of gastrin has been reported to depend partly on the molecular size of the polypeptides

The acid secretion in Heidenhain pouch dogs was followed during instillation of substances of different molecular size and structure in isolated innervated antrum pouches Both polypeptides and some simple amino acids activated the gastrin mechanism The effect of different substances was studied with respect to concentration pH and local anesthesia of the antrum mucosa The lowest concentrations of peptone and glycine causing the corresponding secretion were compared The secretory responses remained high and constant as long as the test substances were left in the antrum pouch For both substances maximal stimulation occurred at pH 7 at pH 5 it was lower and at pH 3 it almost ceased Anesthesia of the mucosa with 2–4 per cent cocain completely abolished the secretory response to peptone and glycine Since a polypeptide and a simple amino acid cause a similar secretory pattern the molecular size seems to be of *secondary importance* Therefore the significance of the chemical structure of the stimulants was investigated The stimulatory power of some simple amino acids dipeptides and some of their derivatives was compared viz glycine sarcosine d and l α alanine β-alanine and glycyl glycine Glycine and β-alanine were the most *active and* caused the same secretory response With sarcosine (N methylated glycine) the secretory response was reduced Both d and l-α alanine caused a low secretory response in contrast to β alanine The effect of glycyl glycine corresponds to that of glycine The findings suggest that the structure of protein compounds especially the position of the amino groups is of importance for the release of gastrin

Eklund, Goran, Curt von Euler and Slawomir Rutkowski (Nobel Institute for Neurophysiology, Karolinska Institutet Stockholm Sweden) PROPRIOCEPTIVE CONTROL OF RESPIRATORY MOVEMENTS

The activity of intercostal muscle spindles is driven largely by their γ-efferent fibres both tonically and rhythmically in phase with respiration Both in the inspiratory and in the expiratory portions of the intercostal musculature the intrafusal muscle fibres of the muscle spindles were activated together with the main extrafusal muscle fibres (*Critchlow* and *Euler* 1963) Rhythmic activation of the intrafusal muscle fibres was driven from supraspinal, probably medullary structures (*Sears* 1963, *Eklund Euler* and *Rutkowski* 1963) Proprioceptive reflexes were able to drive the intercostal γ motoneurones in phase with the movements of the chest wall

A sudden increase of the load of the respiratory muscles by tracheal occlusion augmented the α motoneurone discharge in the vagotomized preparation reflexly This supports the hypothesis that the impulse output from the respiratory integrating mechanism in the medulla to the spinal segments signals the demand for a certain tidal volume, i e for a certain length of the respiratory muscles Changes in the external load of these muscles cause misalignments between intra and extrafusal muscle length which are signalled by the muscle spindle afferents to the α motoneurones (*Eccles, Sears* and *Shaly* 1962) These changes in afferent input to the α motoneurones in turn increase or decrease the power of contraction of the main muscle fibres in such a way that the demand will be achieved in spite of the variations of the external load

Critchlow V and C von Euler J Physiol (Lond) 1963 In press
Eccles R M T A Sears and C N Shealy Nature (Lond) 1962 *193* 844–846
Eklund G., C von Euler and S Rutkowski Acta physiol scand 1963 In press
Sears T A Nature (Lond) 1963 *197* 1013

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Engberg, I (Department of Physiology University of Goteborg Sweden) EFFECTS FROM THE PYRAMIDAL TRACT ON PLANTAR REFLEXES IN THE CAT

It has been postulated that the pyramidal tract exerts an action on motoneurones in the cat by exciting interneurones of various spinal reflex arcs (*Lundberg* and *Voorhoeve* 1962) A reflex excitation of toe extensors (plantar flexors) can be evoked by pressure on the central pad of the cat s hind foot (*Engberg* 1963) Stimulation of the sensorimotor cortex gives excitatory action to the motor nucleus of Flexor digitorum brevis (FDB) By contrast other extensor motor nuclei are inhibited from the cortex in parallel with the strong inhibition of the flexion reflex There is spatial facilitation between the excitatory paths to FDB from the cortex and from the pad It is concluded that impulses in the pyramidal tract excite interneurones of the reflex path from the pad to FDB motoneurones

Lundberg A and P Voorhoeve Effects from the pyramidal tract on spinal reflex arcs Acta physiol scand 1962 *56* 201–219
Engberg I Plantar reflex in cat Experientia (Basel) 1963 In press

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von Euler, U S and F Lishajko (Department of Physiology Karolinska Institutet, Stockholm Sweden) SOME FACTORS DETERMINING THE RELEASE AND UPTAKE OF AMINES IN NERVE GRANULES

Adrenergic nerve granules suspended in isotonic potassium phosphate release noradrenaline at a rapid rate In the presence of noradrenaline 10 µg/ml in the incubation fluid no loss of nor adrenaline is observed Experiments with C^{14} labelled noradrena line indicate that an exchange takes place between labelled and un labelled amine (*Euler Lishajko* and *Stjarne* to be published) Ad dition of adrenaline 10 µg/ml to the incubation fluid does not prevent the loss of noradrenaline from nerve granules

After addition of 1 5–5 mM ATP the noradrenaline content of the granules remains unaltered for a period depending on the pre sence of a sufficient concentration of ATP in the incubation fluid

As shown previously release is greatly retarded by reserpine (10^{-5} M) and phenoxybenzamine (10^{-4} M) and enhanced by tyramine

Uptake of amines in undepleted nerve granules can be shown for adrenaline and dopamine concomitantly with a release of nor adrenaline In partially depleted granules noradrenaline is also taken up

Reserpine does not prevent the amine uptake in partially de pleted granules but prevents the uptake of adrenaline in undepleted granules owing to lack of binding sites

The uptake of amines in partially depleted granules increases when pH is increased from 6 to 8 5 ATP markedly *enhances the* amine uptake and allows an uptake even when amines are present in concentrations of 1 µg/ml or less The ATP-dependent amine *up take* is prevented by reserpine in contrast to the uptake without added ATP

The specificity of the ATP-dependent uptake mechanism is in dicated by the finding that *the uptake of d noradrenaline is small* in comparison with that of the 1 isomer The mechanism of uptake and release will be discussed

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Fenichel, I R and S B Horowitz (Institute of Physiology, University of Uppsala Sweden) THE DIFFUSION OF ORGANIC NONELECTROLYTES IN HYDROGEN BONDING SYSTEMS

If diffusion in cytoplasm proper were the rate limiting process in the cellular transport of simple organic nonelectrolytes a number of recent experimental facts could be reconciled Such diffusion would of necessity show coefficients in the range of 10^{-8} to 10^{-9} cm^2/sec and would manifest marked chemical specificity of the type described by the *Overton-Collander* lipid solubility correlation A study of molecular transport in hydrogen bonded systems has been undertaken to determine whether these requirements are consistent with the known physical properties of cytoplasm The preliminary results of this study are presented The conclusions drawn cast *doubt on the existence of a lipid membrane permeation process* in nonelectrolyte transport and seem to provide insight into the molecular structure of protoplasm

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Fex, J (Nobel Institute for Neurophysiology Karolinska Institutet Stockholm Sweden) CROSSED COCHLEAR EFFERENTS ACTIVATED BY SOUND THROUGH BOTH EARS

The course of the crossed olivo-cochlear fibres was first described in a histological study by *Rasmussen* (1946) When these fibres are electrically stimulated they influence inner ear activity (*Galambos* 1956 *Fex* 1959 1962)

Crossed olivo-cochlear fibres are activated by sound as demonstrated in a previous single fibre analysis (*Fex* 1962) The ear innervated by the fibres under study had been put out of function as part of the preparation Decerebrate cats with both ears intact were used in the present work The activity of single crossed olivo-cochlear fibres was recorded with the microelectrode tips in the midline of the floor of the fourth ventricle The values of the best frequencies (cf *Fex* 1962) for the crossed olivo-cochlear fibres were independent of whether sound was presented to the right ear or to the left The crossed olivo-cochlear fibres could be activated at a lower sound pressure and/or to a higher firing rate through the ear to which they were running than through the opposite ear

Fex J Augmentation of the cochlear microphonics by stimulation of efferent fibres to cochlea Acta oto-laryng (Stockh) 1959 *50* 540–541

Fex J Auditory activity in centrifugal and centripetal cochlear fibres in cat Acta physiol scand 1962 *55* Suppl 189

Galambos R Suppression of auditory nerve activity by stimulation of efferent fibers to cochlea J Neurophysiol 1956 *19* 424–437

Rasmussen G L The olivary peduncle and other fiber projections of the superior olivary complex J comp Neurol 1946 *84* 141–220

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Folkow, B , D Lewis, O Lundgren, S Mellander and I Wallentin
(Department of Physiology University of Goteborg, Sweden)

THE EFFECT OF VASOCONSTRICTOR NERVE STIMULATION ON CONSECUTIVE SECTIONS OF THE INTESTINAL VASCULAR BED

A method which permits continuous and simultaneous recording of the reactions in the resistance and capacitance vessels and in the precapillary sphincters of the intestinal vascular bed, was used to study the effects of regional vasoconstrictor nerve fibre stimulation Both splanchnic nerves were stimulated at frequencies of 1 to 16 impulses per sec During stimulation there was an initial pronounced constriction of the resistance and the capacitance vessels and concomitantly, a decrease of the capillary filtration coefficient (CFC), indicating closure of a number of the precapillary sphincters The maximum response of the capacitance vessels was reached at a lower stimulation frequency than that of the resistance vessels After 30 to 60 sec the resistance and capacitance responses markedly declined in spite of maintained stimulation This decline seemed to be more pronounced for the resistance than for the capacitance vessels at all rates of stimulation For the resistance vessels in turn the extent of this decline was related to the stimulation frequency Thus at high rates (exceeding 4/sec) blood flow in fact returned to the prestimulatory level during maintained stimulation while at lower frequencies the initially induced constrictor response did not fade away entirely On the other hand, the decrease of CFC produced by nerve stimulation was maintained at roughly the same level even if the stimulation period was prolonged for 10 to 15 min When stimulation was stopped there was an immediate and pronounced dilatation of all three vascular sections beyond the prestimulatory level and then a gradual return to normal A marked poststimulatory increase of blood flow was noticed even in those experiments where flow had returned to the resting value already during stimulation

The experiments indicate that vasomotor fibre stimulation evokes a vascular response pattern which is differentiated with regard to the three consecutive sections Local autoregulatory mechanisms seem to modify the neurogenic induced responses par

ticularly within the resistance vascular section The fact that during stimulation CFC is maintained at a decreased value in the face of a gradual restoration of blood flow to normal suggests a redistribution of blood from nutritional vessels to shunts Such a redistribution during stimulation may further be supported by the existence of a poststimulatory hyperemia and a simultaneous increase of CFC above normal

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Folkow, B , D Lewis, O Lundgren, S Mellander and I Wallentin
(Department of Physiology University of Goteborg Sweden)

THE EFFECT OF VASOCONSTRICTOR NERVE STIMULATION ON CONSECUTIVE SECTIONS OF THE INTESTINAL VASCULAR BED

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Abstr XI Scand Physiol Congr Copenhagen *1963*
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Frankenhaeuser, B (Nobel Institute for Neurophysiology Karolinska Institutet Stockholm Sweden) THE IONIC CURRENTS AND THE MEMBRANE ACTION POTENTIAL IN MYELINATED NERVE FIBRES OF XENOPUS LAEVIS

The membrane action potential in the myelinated nerve fibre has been calculated on the basis of the data obtained in voltage clamp experiments (see references below) Figures of the membrane currents and of the action potential will be shown

Dodge F A and B Frankenhaeuser Membrane currents in isolated frog nerve fibre under voltage clamp conditions J Physiol (Lond) 1958 *143* 76–90

Dodge F A and B Frankenhaeuser Sodium currents in the myelinated nerve fibre of *Xenopus laevis* investigated with the voltage clamp technique J Physiol (Lond) 1959 *148* 188–200

Frankenhaeuser B Steady state inactivation of sodium permeability in myelinated nerve fibres of *Xenopus laevis* J Physiol (Lond) 1959 *148* 671–6 6

Frankenhaeuser B Quantitative description of sodium currents in myelinated nerve fibres of *Xenopus laevis* J Physiol (Lond) 1960 *151* 491–501

Frankenhaeuser B Delayed currents in myelinated nerve fibres of *Xenopus laevis* investigated with voltage clamp technique J Physiol (Lond) 1962 a *160* 40–45

Frankenhaeuser B Instantaneous potassium currents in myelinated nerve fibres of *Xenopus laevis* J Physiol (Lond) 1962 b *160* 46–53

Frankenhaeuser B Potassium permeability in myelinated nerve fibres of *Xenopus laevis* J Physiol (Lond) 1962 c *160* 54–61

Frankenhaeuser B A quantitative description of potassium currents in myelinated nerve fibres of *Xenopus laevis* J Physiol (Lond) 1963 In press

Abstr XI Scand Physiol Congr Copenhagen 1963
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Folkow, Bjorn and Eduardo H Rubinstein* (Department of Physiology, University of Goteborg, Sweden) BEHAVIORAL AND GASTROINTESTINAL CHANGES (MOTILITY AND BLOOD FLOW) INDUCED BY ELECTRICAL STIMULATION OF THE LATERAL HYPOTHALAMUS IN CATS

In view of the reported food oriented behavioral activity elicited by stimulation of the lateral hypothalamus the possibility was explored of gastrointestinal changes associated with those stimulus bound effects

Fifteen cats were studied under chloralose anesthesia, recording blood pressure, gastric or intestinal motility or both muscular and intestinal blood flow Stimulation was performed laterally to the ventromedial hypothalamic nucleus using negative square wave *pulses of 1 msec duration 100* cps and *0 05 to 0 7* mA

Two types of autonomic responses were observed *Pattern A* (at the more dorsal points) increased gastrointestinal motility moderate blood pressure increase, marked vasoconstriction in muscle and moderate vasoconstriction in intestine *Pattern B* (at the more ventral points) marked blood pressure increase inhibition of gastrointestinal motility, pronounced vasoconstriction in intestine and moderate vasoconstriction in muscle

Another 12 cats were aseptically implanted with two seven lead electrodes at the same coordinates (A 11, lateral 3) from where the former patterns were induced

Four types of behavioral responses were evoked by the stimulation depending on the dorsoventral height in H C coordinates (H)
Rage (4 cats) typical defense-attack reaction at H -1 to H -3
Exploratory (12 cats) searching activity without eating or drinking at H -2 to H -5,
Alimentary (3 cats) consummatory activity only during stimulation at H -2 to H 4
Flight (3 cats) typical escape reaction at H -5 to H -7

After the behavioral studies the animals were explored under anesthesia recording the same variables as in the first group

In these conditions a correlation was found between the leads that *determined alimentary* and exploratory attitudes and the autonomic pattern A and also a consistent relation between the leads that elicited rage and flight reactions and the autonomic pattern B

* Fellow of the Consejo Nacional de Investigaciones Argentina

So far there is no evidence that the animal makes use of in formation carried by the first impulse but it is tempting to explain the constant sequence of muscle contractions during flight (*Wilson* and *Weis Fogh* 1962) as the result of a stabilizing influence of the exact timing of the first impulse relative to the movement since the timing of motor events becomes irregular after abolishment of the latter Another result of cauterization is a reduction of the flight frequency to 6–8 /sec compared to 17 /sec in the intact animal (*Gettrup* 1962) The sense organs are supposed to couple the me chanical part of the flight system to the central nervous system but experiments failed to prove that the activity is built up within simple reflex arcs (*Wilson* and *Gettrup* 1963)

Armett, C J and R Hunsberger Excitation of receptors in the pad of the cat by single and double mechanical pulses J Physiol (Lond) 1961 *158* 15

Armett, C J J A B Gray R X Hunsberger and S Lal The transmission of information in primary receptor neurones and second order neurones of a phasic system J Physiol (Lond) 1962 *164* 395

Gettrup E Thoracic proprioceptors in the flight system of locusts Nature (Lond) 196? *193* 498

Wilson D M and E Gettrup A stretch reflex controlling wing beat frequency in grashoppers J exp Biol 1963 *40* 171

Wilson D M and T Weis-Fogh Patterned activity of coordinated motor units, studied in flying locusts J exp Biol 1962 *39* 643

Gettrup, Erik (Zoophysiological Laboratory B, University of Copenhagen, Denmark) INFORMATION CARRIED BY SINGLE IMPULSES FROM A UNICELLULAR STRETCH RECEPTOR

In the last few years attention has been paid to the coding of mechanical stimuli in populations of phasic receptors the response of each of which *consists of a single impulse per deformation* (*Armett* and *Hunsberger* 1961, *Armett and al* 1962) A response of this kind does not offer much opportunity for making use of the time dimension and the system was found to operate according to a collective principle, *i e* coding of stimulus intensity by means of the number of active units within the population

Recently, a stretch receptor has been found at the wing hinge *in grashoppers which under the conditions of normal flight fires* only 1–3 times per wing stroke cycle (*Gettrup* 1962) The receptor is of the phasic tonic type and is able to transform stimulus intensity into impulse frequency under tonic as well as under certain phasic conditions However, under natural conditions frequency coding cannot be essential since there is often only one impulse per wing stroke Since the receptor is anatomically isolated, it was of *interest to investigate which information if any, the response could* contain with respect to the deformations normally encountered during flight

The stretch receptor was stimulated phasically in particular by sinusoidal stretching amounting to 50–500 μ The response was analysed with respect to the main parameters of the stimulus At any constant frequency the first impulse of the response always *occurred at the same phase of the stretching movement regardless* of the amplitude of deformation provided that the maximum displacement exceeded 100 μ which is the case in the flying animal (100–200 μ) At smaller deformations (50–100 μ) the phase angle increased with decreasing amplitude Also within the frequency range 5–50/sec, the impulse occurred later as the frequency increased but since the locust has a rather constant stroke frequency this is *of little consequence* to the animal On the other hand the total number of potentials in a response increased with maximum velocity of stretch and of displacement

of 1×10^{-5} to 1×10^{-4} M where they inhibit the transport of sodium a marked fall in oxygen consumption was recorded A part of the O consumption was unaffected by ouabain above this level the oxygen uptake was related to the impulse activity

The effect of other metabolic inhibitors (monoiodo acetate 2-deoxyglucose disodium arsenate, 2–4 dinitrophenol oligomycin chlorpromazine protoveratrine etc) will be discussed together with the effect of different substrates (glucose pyruvate glutamate malate fumarate citrate oxalacetate ketoglutarate lactate) ATP ADP AMP arginine phosphate and phosphate ions on the metabolism of the isolated cell preparation

Chance B P Cohen F Jobsis and B Schoener Intracellular oxidation reduction states *in vivo* Science 1962 *137* 499–508

Giacobini E E Handelman and C Terzuolo *An isolated neurone preparation for studies of metabolic events at rest and during impulse activity* Science 1963 *140* 74

Giacobini E Microdetermination of DPN in single cells 1963 *In press*

Giacobini E E Handelman and C Terzuolo 1963 To be published

Lowry H A quartz fiber balance J biol Chem 1941 *140* 183–186

Giacobini, E (Department of Pharmacology Karolinska Institute Stockholm, Sweden) THE EFFECT OF METABOLIC INHIBITORS ON THE RESPIRATION OF AN ISOLATED NEURONE PREPARATION

Two major approaches have been used to correlate the electrical activity and the metabolism of nervous tissue the manometric measurement of the metabolism of slices of cortex maintained *in vitro* (for references see *McIlwain* and *Rodnight* 1962), and the fluorometric recording of oxidation-reduction levels in the nerve tissue *in situ* (*Chance et al* 1962) A modification of the Cartesian diver technique provides a third possibility An advantage of this technique is that a single living cell can be tested several times under different conditions without apparent damage

This study was performed on the isolated living nerve cell of the slow-adapting crustacean stretch receptor By microdissection the cell was reduced to the minimal dimensions compatible with normal impulse activity (*Giacobini Handelman* and *Terzuolo* 1963) The preparation consisted of the cell body of the neurone (50–75 μ in diameter), a segment of the axon (600 to 750 μ in length) and a portion (400 to 500 μ in length) of the muscle bundle in which the dendrites of the neurone are imbedded The preparation could be kept alive up to 18–24 h and its impulse activity recorded by a metal electrode applied to the axon After the experiments the lipid free dry weight of the cell was determined using a quartz fiber micro balance (*Lowry* 1941)

The technique has been used in studies of respiration, different enzyme activities (respiratory enzymes cholinesterases phospholipases proteinases) and diphosphopyridine nucleotide content (*Giacobini* 1963) Furthermore we have studied the effect on respiration of K Na Ca Cl and Mg all of which modify membrane permeability and polarization (*Giacobini Handelman* and *Terzuolo* 1963)

An increased impulse activity is accompanied by significant changes in the total O uptake (endogenous respiration) suggesting that more than 25 per cent of the cell respiration is related to selective permeability and active transport processes This conclusion is supported by the results obtained with specific inhibitors of transport mechanisms e g ouabain and digoxin In concentrations

reflecting a property of either a recovery process or of some trigger mechanism initiating spontaneous activity On the other hand the relationships of the alpha waves and of the state of wakefulness to temperature described in the literature cannot be purely cortical events Since the activity of the cortex is a function of the tempera ture with a rather flat maximum at body temperature cortical thermosensitivity can hardly be of great importance for thermore gulation

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Gidlof, A and U Soderberg (Nobel Institute for Neurophysiology, Karolinska Institutet *Stockholm, Sweden)* *THE RELATION OF ACTIVITY TO TEMPERATURE IN THE NEURONALLY ISOLATED CEREBRAL CORTEX OF THE CAT*

The electroencephalogram was recorded from neuronally isolated cortical slabs of cats *with brain stem lesions after recovery* from the operation *and anesthesia The slab was large and spontaneously" active in bursts of high frequency waves The frequency* within each burst of activity was constant at all temperatures (25° to 40° C), *and the length of the bursts was largely invariable though* short bursts were slightly more common at the extreme temperatures *Around 38 ° C the interburst intervals were short and of uniform* duration They became longer and more variable with falling tem*perature and also somewhat longer above 38° C From 25° C to* 38° C there was a linear relationship between temperature and the percentage of time taken up by activity The wave amplitudes were constant above 32° C Below this temperature, they declined progressively *In* intact parts of the cortex with uninterrupted connections to remaining regions of the thalamus and hypothalamus sudden changes in activity appeared during heating or cooling They seemed to be related to the effects on the cortical excitatory state that have been seen in the intact brain of animals with unimpaired thermoregulation and were not related with the changes in activity of the isolated cortex *If* the undercut cortex was *not properly* isolated, slow wave activity sometimes invaded the slab over remaining bridges This activity was *also sensitive to temperature* but there was no simple relation between the two variables

The isolated cortex preparation *made it possible to exclude the* effects of anesthesia and the influence from thermoregulatory structures on cortical activity *Further, the electrical activity of the* isolated cortex showed a stereotyped pattern that could be divided into independent variables *such as amplitude and frequency of* waves duration of bursts and periods of silence For these reasons the relation of temperature *to activity could be more readily in*vestigated in this preparation *than in the intact brain The inter*burst intervals showed the *highest temperature dependence thereby*

In agreement with previous observations both excitatory and inhibitory bladder responses were elicited from the first somatic sensory motor area In addition similar effects were obtained from the somatic sensory motor area II (the anterior ectosylvian and the anterior sylvian gyri) Further exclusively excitatory responses were induced from the supracallosal part of the anterior cingulate gyrus and exclusively inhibitory responses from the subcallosal part of the cingulate cortex and the orbital gyrus

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Gjone, R and J Seteklerv (Neurophysiological Laboratory The Anatomical Institute, University of Oslo Norway) INFLUENCE OF CEREBRAL CORTEX ON THE URINARY *BLADDER*

Experimental evidence of bladder contractions elicited from the pericruciate cortex in dogs was presented nearly 90 years ago by *Bochefontaine* Later systematic studies have confirmed his observations and revealed excitatory influence on the bladder activity from several cortical regions Our knowledge about bladder inhibition from the cortical level is more fragmentary probably because the recording technique as a rule has interferred with the sphincter reflex mechanism For the study of bladder inhibition it is of particular importance to have a sufficient background activity and also to include the dynamics of micturition, thus enabling demonstration of interruption of the voiding act

In the present experiments in 22 cats a Nelaton catheter no 12 was inserted into the fundus region through a cystostomy Leaving the urethral outlet fully intact a continuous intravesical pressure recording was established by means of a Statham transducer connected to a Grass polygraph with DC amplifiers After replacing the urine by saline solution at body temperature the bladder volume could be checked through the three way tube connecting the catheter to the transducer

Rhythmic variations of the intravesical pressure were constantly recorded, representing detrusor contractions in response to distension of the bladder *Excitatory* responses to cerebral cortical stimulation included three stages of increased bladder activity (i) augmentation of the amplitude of the rhythmic spontaneous contractions (ii) dribbling urination with incomplete emptying of the bladder and (iii) the complete micturition act *Inhibitory* responses were recorded as (i) diminution or abolition of the rhythmic bladder contractions (ii) interruption of the micturition act and (iii) prevention of the onset of urination Any degree of increased detrusor activity could be produced from all cortical areas yielding excitatory responses Inhibitory stimulation effects were however often significant only when demonstrated by the interruption of urination

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W **Grampp** (Institute of Neurobiology, University of Goteborg Sweden) MULTIPLE SPIKE DISCHARGES OF THE SLOWLY ADAPTING NEURONE OF THE ISOLATED CRUSTACEAN STRETCH RECEPTOR ORGAN

Slowly adapting neurones of isolated lobster stretch receptors fire multiple spike discharges at temperatures above 22–25° C The number of spikes per discharge increases with increasing tempera ture Up to 5–7 spikes, each increase by one in number of spikes per discharge, takes place within an interval of 0 2–0 4° C at tempe ratures, which vary only a few tenths of a degree in any experiment lasting 4–6 hours Transitions from *n* spike to (*n*+*1*) spike dischar ges occur at successively higher temperatures as depolarization of the cell due to strech is increased At higher temperatures the number of spikes per discharge increases rapidly and at 30–33° C most cells fire bursts of several hundred spikes The frequency of bursts de creases but the spike frequency within each burst increases with increasing temperatures approaching 600/sec in bursts lasting about 500 msec All phenomena occurring below 33° C are reversible

Extracellular recording shows that all spikes of a multiple spike discharge are initiated in the initial segment of the axon hav- ing there the same size independent of the frequency within a burst Except for the first, only a few spikes of a high frequency burst reach full height in the soma, most being reduced in size, however all are associated with triphasic extracellular potentials

Extracellular recording from the dendritic region shows diphasic responses with an initial positivity at the same time as the invasion of the soma followed by a slow negativity The latency between soma and dendrite negativity corresponds to an average conduction velocity of 0 1–0 2 m/sec

As temperature increases an afterdepolarization becomes more and more apparent on the initial rapidly falling phase of the spike and may even produce a small hump it precedes the second spike in a two-spike discharge The afterdepolarization as recorded in tracellularly from the soma corresponds in time with the negativity of the extracellular record from the dendrites Simultaneous in tracellular records from the soma and dendritic region show cor respondance in time between soma afterdepolarization and full grown 'dendrite spike'

At high temperatures, when regenerative processes are fast in axon and soma a slow depolarization in the dendritic region may provide the generator potential for multiple discharges in the initial segment of the axon

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Granit, R (Nobel Institute for Neurophysiology, Karolinska Institutet, Stockholm 60, Sweden) TRANSMEMBRANE STIMULATION OF MOTONEURONES TO REPETITIVE ACTIVITY

Together with *Daniel Kernell George K Shortess* and *Richard S Smith* the author has studied the effect of inside stimulation of rat and cat motoneurones through the microelectrode tip in order to measure the quantitative relation between current strength and discharge frequency The results of such measurements will be presented The work has been accepted for publication in full (J Physiol (Lond))

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Guld C (Institute of Neurophysiology University of Copenhagen Denmark) A GLASS COVERED PLATINUM MICRO ELECTRODE

Wolbarsht MacNichol and *Wagner* (1960) described a glass in sulated platinum micro-electrode The method of preparing this electrode has been improved by controlling each step of the proce dure quantitatively

Electrolytic sharpening was controlled by measuring the electro-lyzing current A 50 cps a c voltage was applied between the platinum electrode and a carbon electrode (area > 5 cm) in a 10 M NaCN solution The initial current (about 0 8 A for 0 25 mm pla tinum wire) indicated the depth of the wire in the solution The current decreased as the electrode was etched When the current was about 0 3 A the minimum tip diameter was obtained *i e* about 1 μ for the 0 25 mm electrode Stirring of the solution was not necessary The electrode was cleaned immediately in distilled water and stored in distilled water

The electrodes were coated with 7570 Corning glass by moving the tip of the electrode upward through a molten drop of the glass held in a current heated platinum wire of Ω shape The electrode was transported by a small motor drive at a constant rate of 1 cm/min Coating of the tip of the electrode required a narrow temperature interval A measure of the temperature of the heating wire was ob-tained by measurement of its resistance in a Wheatstone bridge When the tip had passed through the surface of the glass drop (as observed through a microscope) the temperature was gradually increased to ascertain a smooth coating of the tapered tip and of the shatf

The impedance was measured between the platinum core and the outer fluid A completely insulated electrode tip and shaft represents a pure capacitance of less than 2 μμF/mm The capacitance was measured by means of a negative input capacitance with 10 MΩ between input and ground (*Guld* 1962) The coated electrode was stored in air

To remove the glass from the tip of the microelectrode and to platinate the tip the electrode was placed in 0 1 per cent chloro-platinic acid and a negative rectangular pulse of 0 1 sec duration

applied to the electrode Eight to fifteen volt pulses resulted in electrode impedances of about 20 to 1 MΩ The impedance was measured with a ramp impulse of 1 msec duration applied to the electrode through a small condenser (*Guld* 1962)

In cerebral cortex the platinum electrodes found more units per electrode than the glass pipette electrodes, and more of the units had amplitudes larger than 1 mV Metal electrodes with high impedance could hold more units for longer than five minutes than could glass pipette electrodes with high impedance

Wolbarsht M L E F MacNichol Jr and H G Wagner Glass insulated platinum microelectrode Science 1960 *132* 1309–1310

Guld C Cathode follower and negative capacitance as high input impedance circuits Proc IRE 1962 *50* 1912–1927

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Hansson Eskil (Department of Pharmacology Royal Veterinary College Stockholm Sweden) INCORPORATION OF Se SELENOMETHIONINE INTO PANCREATIC JUICE PROTEINS IN VIVO

The concept that protein structure is not absolutely fixed and immutable has been supported by studies on the incorporation of amino acid analogues into proteins Thus the analogues ethionine and p fluorophenylalanine were incorporated into the pancreatic juice proteins (*Hansson* and *Garzo* 1962) It was investigated wheth er selenomethionine could be incorporated in the same way

Se^{75} methionine was prepared by yeast biosynthesis Whole body autoradiography in mice with S^{35} methionine and Se^{75} se lenomethionine showed identical distribution patterns A high up take of the amino acids was observed in protein forming organs like the pancreas the liver and the intestinal mucosa and most of the Se^{75} was found to be associated with the protein fraction Se^{75} selenomethionine was incorporated into the pancreatic juice proteins to the same degree as S^{35} methionine Chromatographic analysis of digested pancreatic proteins showed that the selenom ethionine was incorporated into the pancreatic juice proteins without any prior metabolic changes Pretreatment with methionine reduced the incorporation of selenomethionine indicating a competition be tween methionine and selenomethionine The results suggest that the pancreas can use selenomethionine in place of the sulphur amino acid for syntheses of proteins

Hansson, E and T Garzo Amino acid analogue incorporation into pan creatic juice proteins *in vivo* Biochem biophys Acta 1962 *61* 121–128

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Hesser, C M and G Matell (Laboratories of Aviation and Naval Medicine, Department of Physiology, Karolinska Institutet Stockholm, Sweden) THE ALVEOLO ARTERIAL O DIFFERENCE DURING LIGHT AND MODERATE EXERCISE IN MAN

The alveolar and arterial gas tensions (sitting position) were determined in healthy young male subjects at rest and in the 5th and 6th min of exercise in two series of experiments In the first series (work load 625 kpm/min) the respiratory minute volume and exchange ratio (R) were also determined whereas in the second series (400 and 700 kpm/min) no ventilatory measurements were made in order to avoid interference with free respiration Arterial gas tensions were computed from arterial S_O pH and blood temperature as obtained by continuous analyses The effective alveolar P_O was calculated using the alveolar gas equation substituting arterial for alveolar CO In the first series of experiments the alveolo-arterial P_O difference decreased from a mean value of 14 7 mm Hg at rest to 11 0 mm Hg during exercise In the second series of experiments it was found that, for the range of possible R values the alveolo-arterial P_O difference remained unchanged or decreased somewhat during exercise The decrease in the P_O difference during exercise was due to the fact that arterial P_O showed a larger increment than did alveolar P_O

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Hint, H C (Research Division AB Pharmacia Uppsala Sweden)
SOME FACTORS INFLUENCING THE COLLOID OSMOTIC EFFECT OF DEXTRAN IN THE ISOLATED PERFUSED RABBIT S EAR

The colloid osmotic effect of dextran was studied by weighing the isolated rabbit s ear during perfusion experiments Perfusion with saline or with colloid hypotonic dextran solutions in saline causes edema of the isolated ear Perfusion with colloid hypertonic dextran solutions causes dehydration of the ear and later an edema If perfusion was performed in alternating short periods with hypertonic dextran and saline respectively the ear was held in normal condition for long periods of time Some factors of interest regarding colloid osmotic effects on microcirculation will be discussed

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Haggendal, J and M Lindqvist (Department of Pharmacology, University of Goteborg Sweden) BRAIN MONOAMINE LEVELS AND BEHAVIOUR DURING LONG TERM ADMINISTRATION OF RESERPINE

In previous work we have shown that after the administration of small daily doses of reserpine to rabbits (0 1 and 0 2 mg per kg body weight) the brain levels of noradrenaline, dopamine, and 5-hydroxytryptamine were less than 20 per cent of normal The levels remained low for several weeks, as long as the administra tion continued After every daily dose the rabbits were sedated for some hours with signs of reserpine intoxication About 24 h after the daily reserpine injection, the animals had recovered and looked almost normal The low levels of monoamines in the brain were found 24 h after the daily injection Four hours after the daily dose, when the rabbits showed signs of reserpine intoxication, their brain monoamines were still lower

In rabbits which had received 0 2 mg reserpine per kg body weight daily for eight weeks the brain monoamine levels and the behaviour were studied after the daily injections had been discon tinued From very low levels the monoamines increased slowly and were normal after 4 to 5 weeks After three days, when the mono-amine levels still were low the animals looked normal and con tinued to behave normally The levels of the brain monoamines can thus not be correlated to the behaviour The sedation after reserpine is interpreted as being due to a lack of active brain monoamines

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Hanninen O and K Hartiala (Department of Physiology Univer sity of Turku Finland) ACTIVITIES OF ALKALINE NON SPECIFIC PHOSPHOMONOESTERASE ADENOSINE TRIPHOSPHATE HYDROLYZING ENZYME SYSTEM AND INORGANIC PYROPHOSPHATASE AND CON TENT OF INORGANIC REACTING PHOSPHATE IN THE MUCOSA OF RAT SMALL INTESTINES *

The earlier hypothesis of the phosphorylation mechanism in active hexose absorption has proved untenable However the ac tive absorption mechanisms of nutrients consume energy This energy originates from oxidation of substrates *via* ATP and perhaps other energy rich compounds The upper end of the mucosa of the small intestines contains more ATP than the lower parts (*Hänninen et al* 1963) The concentration of phosphocreatine is highest in the first third and likewise diminishes downwards (*Hanninen* and *Har tiala* 1963) Inorganic phosphate must be available in cells for the free continuation of oxidative phosphorylation

The hydrolytic enzymes studied split esters of phosphoric acid The activity of alkaline nonspecific phosphomonoesterase has its maximum in the very upper end of the small intestines the activity decreases sharply in the first third of the small intestines and then more slowly to about 1/100 of the activity in the upper end The activity of the adenosine triphosphate hydrolyzing enzyme system has its maximum likewise in the upper end but the activity di minishes more slowly downwards and about 50 per cent of the upper end activity is still left at the mid point of the small intestines In the lower end of the small intestines the activity is less than one fourth of the upper end activity The activity of inorganic pyro phosphatase remains about the same in the first sixth of the length of the small intestines and then the activity decreases roughly linearly to about one fifth of the upper end activity in the lower end The content of inorganic reacting phosphate is about 10 µmoles g dry weight and is about the same in different regions of the small intestines

Possibly the mucosal cells have a certain urge to maintain a

This study has been supported by the Sigrid Jusélius Foundation

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In previous work we have shown that after the administration of small daily doses of reserpine to rabbits (0 1 and 0 2 mg per kg body weight) the brain levels of noradrenaline, dopamine, and 5 hydroxytryptamine were less than 20 per cent of normal The levels remained low for several weeks as long as the administration continued After every daily dose the rabbits were sedated for some hours with signs of reserpine intoxication About 24 h after the daily reserpine injection, the animals had recovered and looked almost normal The low levels of monoamines in the brain were *found 24 h after the daily injection Four hours after the daily* dose, when the rabbits showed signs of reserpine intoxication, their brain monoamines were still lower

In rabbits which had received 0 2 mg reserpine per kg body weight daily for eight weeks the brain monoamine levels and the behaviour were studied after the daily injections had been discontinued From very low levels the monoamines increased slowly and were normal after 4 to 5 weeks After three days, when the monoamine levels still were low, the animals looked normal and continued to behave normally The levels of the brain monoamines can thus not be correlated to the behaviour The sedation after reserpine is interpreted as being due to a lack of active brain monoamines

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Jacobsohn, Dora (Institute of Physiology University of Lund Sweden) EFFECTS OF TESTOSTERONE AND RELATED SUBSTANCES ON THE DEVELOPMENT OF REPRODUCTIVE FUNCTIONS IN FEMALE RATS

Recent investigations have shown that female rats given one injection of testosterone during the neonatal period become permanently sterile Vaginal opening occurs earlier than normally Vaginal smears indicate constant oestrus Numerous large follicles but no corpora lutea are present in the ovaries Different investigations indicate that testosterone interferes in an unknown manner with the differentiation of cerebral structures necessary for the cyclic production or release of luteinizing hormone (LH) in the pituitary gland The previous studies have been carried out on rats of mature age (*Barraclough* and *Gorski* 1961 *Takewaki* 1962)

In the present work testosterone was injected into 5-day-old rats and the reproductive organs were studied from the 10th day until about 2 months of age The ovaries of the injected rats were smaller than those of litter mate controls The impairment of ovarian growth observed before puberty indicates that testosterone affected the output of luteinizing hormone and of other gonadotrophic hormones A direct inhibitory effect of testosterone on the ovary cannot be excluded Other substances with biological actions related to those of testosterone (17β-hydroxy 17α methyl 1 4 androstadiene 3-one 19 nor testosterone 17β-phenylpropionate and 1-methylΔ^1 5-α androstene 17β-ol 3-one 17β-enanthate) were tested in a similar way and in addition vaginal smears were taken during 2 to 3 months The rats were then caged together with intact males and births and nursing of young were recorded

Barraclough C A and R A Gorski Endocrinology 1961 68 68
Takewaki K Experientia 1962 *18* 1

constant concentration of inorganic phosphate To make this possible, the upper end of the small intestines would need more hydrolytic enzymes for its higher metabolic rate The distribution of the activity of inorganic pyrophosphatase in duodenal mucosa may signify a uniform distribution of some synthesizing inorganic phosphate producing processes in this region and that these syntheses decrease almost linearly towards the lower parts of the small intestines

Hanninen O K Hartiala and V Nurmikko 1963 To be published
Hanninen O and K Hartiala 1963 To be published

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Jahn, Samia Al Azhana (Institute of Neurophysiology University of Copenhagen Denmark) PHYSIOLOGICAL PROPERTIES OF ISOLATED NEUROMUSCULAR SPINDLES

The response to mechanical deformation of isolated muscle spindles from the frog was studied by leading off action potentials from the equatorial zone The amplitude and rate of deformation could be varied independently and the movements of the driving system were correlated with microscopical measurement of the dis placement of a graphite granule placed on the surface of the equa torial zone

There exists a minimum deformation of $8 \pm 2\ \mu$ which must be exceeded in order to elicit a response The threshold deformation is independent of the deformation rates (0 1–6 5 mm/sec) and of the initial elongation (10–20 per cent) This observation agrees with those obtained from Pacinian corpuscles (*Gray* and *Matthews* 1951) and amphibian touch receptors (*Hoglund* and *Lindblom* 1961)

With threshold deformation and deformation rates of 4–6 5 mm/sec the latency of the response was 6 msec (20 C) The mini mum latency of 3 msec was obtained with deformation rates higher than 7 mm/sec and deformations exceeding 3 times threshold With deformations exceeding 2 times threshold double firing occurred, the interval between responses decreasing with increasing rate of deformation to a minimum interval of 2 msec

The excitability of the spindle was investigated by applying paired stretches of equal size With threshold deformation and stretch durations of 2 and 5 msec and a time interval between the two stretches of the pair exceeding 40 and 50 msec respectively there was a response to every test stimulus With decreasing intervals not every test stimulus evoked a response with an interval of 25 and 40 msec respectively half of the test stimuli evoked a response For intervals of less than 15 msec there was no longer a response With deformations of 5 times threshold and a stretch duration of 2 msec each test stretch evoked a response when the interval be tween stretches was 6 msec or more the latency of the test response being delayed by 1 msec

When exposed to a train of deformations (duration 3 sec) the maximum frequency at which the spindle still responded to each

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Jacobsson, S and I Kjellmer (Department of Plastic Surgery, University Hospital Malmo, and Department of Physiology, University of Goteborg Sweden) RELATION BETWEEN LYMPH FLOW AND ACCUMULATION OF CAPILLARY FILTRATE IN EXERCISING MUSCLE

Lymph flow from resting calf muscles of cats was found to be *negligible* Standardized passive movements of the ankle joint produced a lymph flow of 0 03 *ml/min/100 g muscle* With superimposed muscular contractions lymph flow increased to 0 05 ml/min/ 100 g muscle This flow amounts to 10 to 20 per cent of the capillary filtration rate in exercising muscle, but seems great enough to return *the proteins of the capillary filtrate to the blood circulation* Since lymph flow cannot keep pace with the formation of capillary filtrate an accumulation of fluid occurs in the exercising muscle With time the rate of net outward capillary filtration declines and finally stops

The outward filtration during muscular exercise is due to a rise in capillary hydrostatic pressure upsetting the Starling equilibrium' The fact that outward filtration gradually declines implies a restoration of this equilibrium A return of hydrostatic capillary pressure to normal during maintained exercise seems unlikely, while an increase of the effective colloid osmotic pressure gradient across the capillary membrane could to some extent contribute to the limitation of outward filtration The main limiting factor, however, must be an increase of tissue pressure

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Jansen, Jan K S and Torstein Rudjord (Neurophysiological Laboratory The Anatomical Institute University of Oslo Norway) THE SILENT PERIOD DURING TWITCH CONTRACTION OF THE SOLEUS OF THE DECEREBRATE CAT

The silent period during contraction of skeletal muscles has been investigated repeatedly Three different spinal mechanisms *contribute to a varying degree* to this phenomenon (*1*) the recurrent Renshaw inhibition (early part of silent period) (2) the pause of the excitatory Ia afferents from the muscle (*3*) the inhibition due to activation of the Golgi tendon organs As emphasized by *Merton* (1951) the duration of the silent period is of particular importance for the control of movement Recently *Hufschmidt* (1960) claimed that the duration of the silent period during voluntary activity is determined by the inhibitory effects of tendon organ afferents This prompted the present reinvestigation in the decerebrate cat as a study of the silent period under isometric and nearly isotonic twitches would differentiate between the effects of the spindle pause and the tendon organ inhibition

The experiments were performed on intercollicularly decerebrated cats with sustained stretch reflexes in soleus The electromyogram of the soleus was recorded by belly tendon leads The background activity was the stretch reflex produced by varying degrees of static extension of the muscle by a pull to its isolated tendon The muscle tension was recorded by an isometric myograph Twitches superimposed on the steady stretch reflex were produced by nerve stimulation of intensities from α threshold to α maximum Nearly isotonic conditions were obtained by the insertion of weak springs between the muscle and myograph

In different preparations the duration of the silent period varies considerably (80–200 msec) it remains fairly stable however in any one preparation The level of initial tension does not appreciably influence the duration of the silent period Under isometric conditions the increase in tension during a twitch elicited by an α maximal shock was usually *several hundred* grams With a weak spring in series the muscle shortens and the tension increment during the twitch might be only 2–3 per cent of the isometric twitch tension

stimulus was 16–20 stretches per second at threshold and 60–70 per second with deformations of 2 times threshold

A spontaneous contraction of intrafusal muscle fibres (*Buchthal* and *Jahn* 1957) caused a transient increase in responsiveness of the spindle to frequencies which otherwise were ineffective in evoking a response to every stimulus

Buchthal F and U Jahn Spontaneous activity in isolated muscle spindles Acta physiol scand 1957 *42* Suppl 145

Gray J A B and P B C Matthews A comparison of the adaptation of the Pacinian corpuscle with the accommodation of its own axon J Physiol (Lond) 1951 *114* 454–464

Hoglund G and U Lindblom The discharge in single touch receptors elicited by defined mechanical stimuli Acta physiol scand 1961 *52* 108–119

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Jansson Gunnar and Jan Martinson (Department of Physiology University of Goteborg Sweden) RELEASE OF A PLASMA KININ FORMING ENZYME FROM THE STOMACH OF THE CAT

Recent findings indicate that two different types of efferent vagal nerve fibres affect gastric functions (*Martinson* 1962 *Martinson* and *Muren* 1963) These two efferent fibre groups have different stimulation thresholds and can therefore be stimulated more or less selectively Fibres with relatively low stimulation threshold *increase gastric motility whereas fibres with higher stimulation* threshold elicit secretion of HCl and pepsin The latter fibre group also evokes vasodilatation in the stomach and inhibition of gastric motility These effects might be elicited by a plasma kinin mechanism of importance for the functional vasodilatation during secretory activity as proposed for other glandular tissues (*Hilton* 1960)

The plasma kinin forming activity of neutralized gastric juice and of Tyrode solution perfused through the gastric vascular bed has been tested with the method described by *Hilton* and *Lewis* (1956)

Gastric juice contains a plasma kinin forming enzyme with properties of kallikrein Perfusate collected during stimulation of the high threshold fibres of the vagus contains small amounts of a similar or identical enzyme Further quantitative and qualitative aspects concerning this enzyme will be presented

Hilton S M M Schachter Polypeptides which affect smooth muscles and blood vessels Pergamon Press 1960 258–262
Hilton S M and G P Lewis J Physiol (Lond) 1956 *134* 471–483
Martinson J Intern Congress Series 196? *48* 381
Martinson J and A Muren Acta physiol scand 1963 *57* 309–316

Even so, the total duration of the silent period was not changed greatly The peak time of the "isotonic" twitches (90 msec) was however, appreciably longer than the isometric ones (70 msec) indicating that the motoneurones resume firing earlier during the falling phase of the "isotonic" twitches

Thus, these experiments indicate that the Golgi tendon organs do not play an essential part in the determination of the duration of the silent period Presumably the pause of Ia spindle afferents is the most important factor

Hufschmidt H J Pflugers Arch ges Physiol 1960 *271* 35–39
Merton P A J Physiol (Lond) 1951 *114* 183–198

found in the brain and skin of normal rats Single injections of morphine into normal rats both in toxic and analgesic doses , did not alter the histamine content in the brain or in the skin

The *in vitro* experiments show that morphine liberates histamine only when very high concentrations are applied Therefore neither the development of morphine tolerance nor the analgesic action of morphine in rats is connected with liberation of histamine

Mongar I L Measurement of histamine releasing activity In Histamine Ciba Found Symp Churchill London 1956 p 74–91

Abstr XI Scand Physiol Congr Copenhagen 1963
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Johannesson, Torkell and Svend Norn (Department of Pharmacology, University of Copenhagen, Denmark) THE INFLUENCE OF MORPHINE ON THE HISTAMINE CONTENT IN THE BRAIN AND SKIN OF THE RAT

Morphine has been considered to liberate histamine (*Mongar* 1956), we have investigated its effect on the histamine content of rat brain and skin Furthermore, the histamine liberating capacity of Compound 48/80 Polymyxin B and morphine was studied *in vitro* in a suspension of mast cells derived from the rat peritoneum

For the histamine analyses we used the whole brain (excluding the pituitary gland) and approximately 1 g of skin which, after depilation, was excised from the forepart of the back of the animal Histamine was coupled to dinitrofluorobenzene and determined photometrically

Morphine tolerance in the rats was produced by daily subcutaneous injections of morphine in increasing doses for 25 days The last daily dose was about 150 mg/kg

Twenty one morphine tolerant rats were killed 48 hours after the last daily dose of morphine For comparison, 21 normal rats were killed without any previous administration of drugs The histamine content in the brain of rats in the tolerant animals averaged 8 0 μg/g (S D = 1 2) and in the control group 8 0 μg/g (S D = 0 8) In the skin the concentration averaged 31 μg/g in the tolerant rats (S D = 7) and 27μg/g in the normal rats (S D = 5)

Single intraperitoneal injections of 150 mg/kg or 8 mg/kg morphine into normal rats did not alter the histamine content in the brain or in the skin either when the animals were killed 48 hours or 30 minutes after the injections

When Compound 48/80 and Polymyxin B (0 5 μg/ml) were added to the mast cell suspension Compound 48/80 liberated 72 per cent and Polymyxin B 66 per cent of the total amount of histamine in the suspension In a concentration of 100 μg/ml morphine released 39 per cent of the total amount of histamine in the suspension When the morphine concentration was increased to 1000 μg/ml, all histamine was released

The results indicate that the amounts of histamine in the brain and skin of morphine tolerant rats are not different from those

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Kaada B R, F Thomas, E. Alnæs and K Wester (Neurophysiological Laboratory The Anatomical Institute University of Oslo Norway) SYNCHRONIZATION IN THE ELECTROENCEPHALOGRAM (EEG) INDUCED BY HIGH FREQUENCY STIMULATION OF THE MIDBRAIN RETICULAR FORMATION IN ANESTHETIZED CATS

High frequency stimulation of the brain stem reticular formation produces a generalized desynchronization of the electrocortical waves *Low* frequency stimulation of the same structures may under certain conditions induce EEG synchronization mainly consisting of increased spindle activity

In a study of the EEG effects of midbrain electrical stimulation in anesthetized cats it was discovered that *high* frequency stimulation occasionally produced an immediate and widespread increase in cortical slow wave activity The conditions for obtaining this type of synchronization have been investigated Further the response has been mapped with respect to its origin in the brain stem and to its cortical distribution

The synchronizing effect in response to high frequency stimulation was present only in animals under a relatively light anesthesia In non anesthetized cats either curarized or after postcollicular transection high frequency midbrain reticular stimulation resulted in the typical desynchronization with fast activity of low amplitude Under light chloralose urethane or Nembutal® anesthesia desynchronization by reticular stimulation was frequently replaced by (i) regular slow waves of high amplitude and of frequency 1–4/sec (ii) a marked increase and regularization of the randomly occurring chloralose waves or (iii) sometimes an increase in spindle activity At still deeper anesthesia the threshold for obtaining synchronization increased and finally the response disappeared

The optimal stimulation frequency for producing synchronization under anesthesia was around 100–300/sec but clear-cut effects were usually also obtained at frequencies down to about 30/sec and occasionally at rates as low as 10/sec

EEG synchronization under anesthesia was elicited from the entire reticular formation of the midbrain which was explored in steps of one millimeter with bipolar stimulating electrodes At

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Johansen, S H , Mogens Jørgensen and Sv Molbech (Department of Anaesthesia, and the Cardio Respiratory Laboratory, Gentofte County Hospital, and Danish National Association for Infantile Paralysis, Hellerup, Denmark) EFFECT OF CURARE ON RESPIRATORY AND NON RESPIRATORY MUSCLE POWER IN MAN

The ability of patients to lift their heads postoperatively when neuromuscular block had been induced during anaesthesia is commonly used as a test of restitution of muscle power Neuromuscular block was produced by tubocurarine, and muscular depression in respiratory power and in hand grip strength related quantitatively to the ability of raising the head

In six experiments on five subjects (20–23 years old) the course of restitution after maximal depression of head lifting was compared with restitution of the hand grip and the maximal inspiratory and expiratory flows To obtain isometric conditions in all of the three muscle groups examined measurements of maximal inspiratory and expiratory pressures replaced those of maximal flows in four experiments on four subjects In all experiments the respiratory functions were less affected than head lifting and hand grip head lifting being most affected

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Kjellmer, Ingemar (Department of Physiology University of Gote borg Sweden) AN INDIRECT METHOD FOR ESTIMAT ING TISSUE PRESSURE IN SKELETAL MUSCLE

Direct measurement of the hydrostatic pressure in the interstitial fluid is impossible because of the minute dimensions of the spaces and the gel character of the fluid Since tissue pressure is one of the determinants of capillary filtration and since it might be a major limiting factor for filtration exchange during muscular exercise it was considered of interest to develop a method for indirect estima tion of tissue pressure

The method is based on the following considerations In col lapsible vessels the intravascular pressure must be at least as high as the extravascular pressure to permit passage of blood The veins are collapsible vessels and therefore the pressure inside patent veins can never be less than tissue pressure If the tissue pressure is 10 mm Hg the pressure in the veins inside the organ (local venous pressure LVP) is at least 10 mm Hg in spite of the fact that the pressure in the veins just outside the organ (central venous pressure CVP) may be considerably lower Hence a steep pressure drop can exist where the vein leaves the boundaries of the organ described as the waterfall phenomenon (*Permutt et al* 1961) This implies that a rise *of CVP does not influence LVP until CVP has reached* tissue pressure Thus the level at which a gradual rise of CVP be gins to increase LVP is used to estimate the tissue pressure Since the veins constitute the main capacitance vessels even a slight in crease of LVP produces an increase in regional blood volume In these experiments the volume of the calf of the cat was recorded with a plethysmograph permitting accurate measurement of even small changes of regional blood volume CVP was measured and could be increased stepwise The estimated tissue pressure during rest was close to zero and rose during exercise by 5–10 mm Hg

Permutt, Bromberger Barnea and Bane (1961) *cit in Riley R L* Effect of lung inflation upon the pulmonary vascular bed Ciba Foundation Symposium on Pulmonary Structure and Function Churchill Ltd Lon don 1962

caudal levels a low threshold area was found in the midline in the region of the decussation of the brachium conjunctivum The response was, however, independent of this structure, being present also in chronic decerebellectomized cats At rostral levels of the midbrain the optimum zone was located ventrolateral to the central grey substance When present on midbrain stimulation EEG synchronization could also be produced by high frequency stimulation of thalamic midline or intralaminar nuclei

Synchronization in response to high frequency reticular stimulation was recorded from the entire neocortex and the slow waves were bilaterally synchronous Depending on the site of stimulation the greatest amplitude was frequently recorded over the parieto-occipital or anterior cortical regions The same stimulus sometimes produced synchronization in some leads and desynchronization in others The response was absent in the hippocampus

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Permutt Bromberger Barnea and Bane (1961) cit in *Riley R L* Effect of lung inflation upon the pulmonary vascular bed Ciba Foundation Symposium on Pulmonary Structure and Function Churchill Ltd Lon don 1962

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Kjellmer, I and H Odelram (Department of Physiology University of Goteborg, Sweden) COMPARATIVE EFFECTS OF HISTAMINE, BRADYKININ AND ACETYLCHOLINE ON CAPILLARY FILTRATION IN SKELETAL MUSCLE

Histamine and bradykinin, proposed to act as vasodilator substances under physiological conditions, increase capillary permeability when applied in fairly high concentrations intradermally It was investigated whether a change of capillary permeability occurred even when the two substances were infused intraarterially in amounts which just barely produced a maximum vasodilatation

The experiments were performed on the calf muscles of cats Regional blood flow, controlled with a constant perfusion pump, perfusion pressure and muscle volume were recorded With this technique the capillary filtration coefficient (CFC) could be determined Variations in CFC reflect changes of capillary permeability and size of capillary surface area Acetylcholine was used as a reference substance since it was considered to increase CFC only by relaxing precapillary sphincters and thus enlarging capillary surface area

Acetylcholine raised CFC two to three times With equivasodilating doses histamine and bradykinin produced a greater increase of CFC than acetylcholine Histamine and bradykinin in doses producing a large increase of CFC caused a swelling of the muscle, due to a net outward fluid transfer This swelling was irreversible, indicating an escape of plasma proteins into the extravascular compartment Contrary to this acetylcholine produced only a slight and transient increase in volume

Thus both bradykinin and histamine increase capillary permeability even in such low doses as barely elicit maximum vasodilatation If these substances are physiological vasodilators it then remains to be explained how they can act without producing tissue oedema owing to leakage of plasma proteins

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Klausen, K (Laboratory for the Theory of Gymnastics University of Copenhagen Denmark) CARDIAC OUTPUT IN EXERCISE DETERMINED BY A CO METHOD AND THE ACETYLENE METHOD A COMPARATIVE STUDY

In 1961 *Defares et al* described a new CO_2-method for the determination of cardiac output The purpose of the present experiments was to study the applicability of this method during exercise and to compare it with the acetylene method

The work experiments were performed on the *Krogh* bicycle ergometer After a steady state had been reached the O_2-uptake and the CO_2-output were determined by the *Douglas* bag method The CO method was performed as follows At the end of a deep expiration the subject was connected by means of a three way stopcock to a *Grollman* bag containing 2–2 5 1 of an oxygencarbon dioxide mixture (about 6 per cent CO and 94 per cent O) and rebreathed for a period not exceeding 8–12 sec depending on the work level Samples of air from the bag were taken during each inspiration by means of evacuated sampling tubes The respiratory cycles were registered by a strain gauge The samples were analysed on *Scholander*s gas analysis apparatus The CO content in the five samples was plotted against time and the best fitting line drawn The abscissa was subdivided into equal arbitrary time intervals and the corresponding CO_2-percentages converted into CO_2-tensions (P_{CO_2}) The first P_{CO_2} was then plotted against the second the second against the third etc After the first few points a straight line was obtained and the point of intersection between this line and the 45 line gave the mixed venous P_{CO} The arterial P_{CO} was assumed to be equal to the alveolar P_{CO} The latter was calculated using the *Bohr* formula and a physiological dead space estimated according to *Asmussen* and *Nielsen* (1956) The CO_2 content of arterial and venous blood was determined from a standard CO dissociation curve of oxygenated blood The CO_2-output divided by the venous arterial CO_2-difference gives the cardiac output At the end of each experiment the acetylene method was applied The results determined by this method and the CO method were almost identical

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Kjellmer, I and H Odelram (Department of Physiology University of Goteborg Sweden) COMPARATIVE EFFECTS OF HISTAMINE, BRADYKININ AND ACETYLCHOLINE ON CAPILLARY FILTRATION IN SKELETAL MUSCLE

Histamine and bradykinin, proposed to act as vasodilator substances under physiological conditions, increase capillary permeability when applied in fairly high concentrations intradermally It was investigated whether a change of capillary permeability occurred even when the two substances were infused intraarterially in amounts which just barely produced a maximum vasodilatation

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Knutsson, E. and C R. Skoglund (Department of Physiology Karolinska Institutet Stockholm Sweden) RESPONSES OF FROG MUSCLE FIBERS TO SLOWLY RISING CURRENTS

Linearly rising currents of different gradients were applied to frog sartorius muscles by a capillary electrode inserted at the nerve free end of the muscle and the responses were recorded by another internal electrode placed about 50 μ farther away The firing level spike amplitude and stimulus threshold were analyzed under different experimental conditions the subthreshold membrane potential changes will be dealt with in a separate work by *Knutsson* (1963)

In 95 per cent of the fibers the responses were uniform as follows The firing level as determined by different methods was found to be constant over a wide range of stimulus gradients corresponding to spike latencies up to 1 sec At latencies of more than 40 msec the spike amplitude was successively reduced with diminishing rates of rise of the stimulating current while the spike duration remained unaffected The threshold of stimulation in terms of current strength at the moment of spike discharge decreased with diminishing stimulus gradient (negative accommodation cf *Skoglund* 1945)

In a series of experiments the membrane potential was preset at various levels above and below the resting potential Hyperpolarization of up to 40 mV did not affect threshold firing level or spike amplitude of the spikes elicited by linearly rising currents Depolarization caused a shift of the firing level towards zero with diminishing stimulus gradients the spike amplitude decreased and the spike duration increased resulting in graded responses At a certain minimal gradient the spike mechanism failed completely and only rectification was observed

Increase of the extracellular Ca-concentration resulted, *i a* in a change of the critical membrane potential towards zero level (30 mV at 30 mEq/l) and in the occurrence of various types of graded spike responses comparative studies of the effects of a decreased Ca-concentration were also made

The atypical responses to slowly rising currents found in 5 per cent of the fibers consisted in graded responses and the occurrence

Asmussen E and M Nielsen Physiological dead space and alveolar gas pressures at rest and during muscular exercise Acta physiol scand *1956* *38* 1–21

Defares J G M E Wise and J W Duyff New indirect Fick procedure for the determination of cardiac output Nature (Lond) *1961* *192* 760–761

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Koivikko, A., T Peltonen and L Hirvonen (Department of Physiology Turku University Finland) BLOOD CIRCULATION TIME IN DOGS OF DIFFERENT AGES

Adaptation of the circulation after birth is not complete after closure of the foetal short circuits Studies on the blood circulation time at different ages generally do not include simultaneous determination of blood volume and cardiac output. The present investigation includes studies of 18 dogs of different ages from 1–12 months Blood circulation time from the femoral vein to the paw of the hind leg was determined with fluorescein Blood volume was measured by the dye dilution technique and cardiac output by the direct Fick method The blood circulation time increased with increasing age body weight length of animal (from the anterior tip of the sternum to the paw of the hind leg) blood volume and cardiac output the correlation to these factors being somewhat irregular When circulation time was plotted against the ratio of blood volume to cardiac output a linear relationship was obtained

of a minimal gradient In most cases the phenomena could be referred to a pre-existent depolarization, on some occasions slow muscle fibers may have been impaled

Knutsson E 1963 To be published
Skoglund C R Kungl Sv Vet Akad Handl 1945 *21* Serie III No 9

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Kruhøffer P and O I Nissen (Institute of Medical Physiology University of Copenhagen Denmark) HANDLING OF GLYCEROL IN THE KIDNEY

In experiments in cats the tubular reabsorption of glycerol was found to be essentially complete (and the urine/plasma ratio less than one) at low filtered loads At increasing filtered loads the rate of reabsorption continued to rise and the reabsorption fraction tended to converge to a limiting value of about 0 4

In some experiments simultaneous determinations were made of the rate of metabolic conversion of glycerol in the kidneys a differential pressure flowmeter introduced without interrupting the renal blood circulation was used to measure the renal (venous) blood flow (RBF) The rate of conversion was calculated a RBF (arterial glycerol conc − renal venous glycerol conc) − rate of glycerol excretion This rate was found to rise to an apparent maximum with increasing arterial plasma concentrations up to about 150–300 mg/l and at this saturation level it amounted to about 20 per cent of the rate of conversion in the body as a whole or to about 0 09 mg per minute per gram of kidney At low filtered loads the rate of conversion exceeded the reabsorption rate but at high loads (corresponding roughly to arterial plasma concentrations of 500–1000 mg/l) the relation is undoubtedly reversed Stop-flow experiments indicated that the most distal parts of the nephron are sparsely permeable to glycerol and that concentrations below that of arterial plasma may arise in proximal parts

The observations may be accounted for on the assumptions that reabsorption at low filtered loads is predominantly a conversion reabsorption (diffusion from the tubular lumen into tubular cells maintained by a metabolic conversion inside the cytoplasm) and that at high filtered loads well above the level of saturation of the glycerol converting enzymes it is essentially due to transcellular back-diffusion through the (proximal) tubular cells

The pattern of renal excretion of glycerol may thus be interpreted without resorting to special transmembrane transfer mechanisms

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Krog, John and Ole B Reite (Institute for Experimental Medical Research Ulleval Hospital, Oslo, Norway) THE CIRCULATORY EFFECT OF BRADYKININ IN BIRDS

The circulatory effect of bradykinin in birds was investigated in domestic ducks and chickens Intravenous infusion of 1 μg of synthetic bradykinin caused only small variations in blood pressure This is in contrast to the effect in mammals from a similar dosage, where a marked fall in blood pressure was obtained The possible causes of this difference will be discussed

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Larsen Valdemar (Dumex Ltd Copenhagen Denmark) THE TERATOGFNIC EFFECTS OF THALIDOMIDE IMIPRAMINE HCL AND IMIPRAMINE N OXIDE HCL ON WHITE DANISH RABBITS

Forty female rabbits of the white Danish breed were used in this investigation After mating 14 were given thalidomide daily for a certain period 10 received imipramine HCl 10 received imipramine N-oxide and 6 controls received saline solution All injections were subcutaneous

Macroscopic examination showed that 22 of 35 young of thalidomide treated rabbits were abnormal in some way one of 53 young of imipramine treated rabbits and one of 59 young of imipramine N-oxide treated rabbits were abnormal while none of the 25 young from the control group was abnormal The effect of thalidomide depended on the time elapsed after mating and start of administration Thalidomide in a dose of 100 mg/kg markedly reduced the fertility while imipramine HCl or imipramine N-oxide (15 mg or 25 mg per kg daily) did not reduce fertility Thus, the white Danish rabbit can be used in the search for teratogenic effects of thalidomide like substances

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Lang, H (Department of Physiology, University of Turku Finland) TEMPORAL ASPECTS OF THE GALVANIC SKIN RESPONSE (GSR)

In determinations of the GSR (a sweat gland reaction controlled by the sympathetic nervous system), main attention has been paid to its amplitude When recording the GSR by the *Tarchanoff's* potential method with the use of a DC amplifier, marked variations have been observed in the duration of the GSR evoked by local stimulation of the cat s brain Before quantitative studies can be made on the temporal properties of the centrally evoked GSR, it is necessary to determine the laws controlling the long lasting GSR evoked by peripheral efferent nerve stimulation

By repetitive direct electrical stimulation of the lumbal sympathetic chain, a long-lasting negative skin potential can be recorded from the central pad of the cat s ipsilateral hind limb Shocks applied at a rate of about 2/sec produced complete fusion of the GSR This fused or tonic GSR had the following characteristics After a short latency there was a rapid rising phase of 2–6 sec duration during repetitive stimulation there was a pronounced slow linear decrease, and, after termination of the stimulation, the potential decreased roughly exponentially, slowly approaching the base line The maximum of the frequency-response curve was obtained at a stimulus rate of 3–25/sec

The drop of the GSR during repetitive stimulation of *e g* 20 sec averaged 30 per cent of its peak amplitude This represents the greatest tonicity of the GSR which the peripheral gland mechanism on an average is able to produce The effect of (*i*) the intensity of the stimuli, (*ii*) their frequency and (*iii*) of the interval between trains of stimuli on the decline of the GSR, was analysed

In summary, by its sensitivity speed of reactions and regularity of shape the GSR represents a useful autonomic criterion also for the temporal properties of the efferent action of the central nervous system Preliminary results will be presented regarding the GSR of varied tonicities evoked by differently localized central stimulations (see abstract, p 157, this volume)

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Larsen, Valdemar (Dumex Ltd Copenhagen Denmark) THE TERATOGENIC EFFECTS OF THALIDOMIDE IMIPRA MINE HCL AND IMIPRAMINE N OXIDE HCL ON WHITE DANISH RABBITS

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Macroscopic examination showed that 22 of 35 young of thalidomide treated rabbits were abnormal in some way one of 53 young of imipramine treated rabbits and one of 59 young of imipramine N-oxide treated rabbits were abnormal while none of the 25 young from the control group was abnormal The effect of thalidomide depended on the time elapsed after mating and start of administration Thalidomide in a dose of 100 mg/kg markedly reduced the fertility while imipramine HCl or imipramine N-oxide (15 mg or 25 mg per kg daily) did not reduce fertility Thus the white Danish rabbit can be used in the search for teratogenic effects of thalidomide like substances

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Lichtneckert, S and G Lundin (Institute of Physiology, University of Lund, Sweden) DEMONSTRATION OF NON VENTILATED PARTS IN NORMAL LUNGS

The nitrogen elimination technique was used to determine the functional residual capacity The use of maximal tidal volumes mobilized a nitrogen volume not usually involved in ventilation This amount of trapped air varied with body posture and age of the subjects

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Lowy, J and O Sten Knudsen (Institute of Neurophysiology University of Copenhagen Denmark) LATENCY RELAXATION IN INVERTEBRATE MUSCLES

Latency relaxation *i e* the small drop in tension which precedes isometric contraction was first observed in frog muscle (*Rauh* 1922 *Sandow* 1944) and later in other vertebrate skeletal muscles (*Abbott* and *Ritchie* 1951) Under conditions resembling those under which latency relaxation occurs in vertebrate muscle *Abbott* and *Ritchie* (1956) were unable to demonstrate a latency relaxation in certain invertebrate muscles We have studied the same invertebrate muscles with improved technique and found a latency relaxation in all of them

In the obliquely striated cephalopod muscles (the funnel retractor of *Loligo* and *Octopus*) latency relaxation was only present over a narrow region of muscle lengths *i e* up to 1 15 times equilibrium length The latency duration and amplitude of latency relaxation were almost identical with those in frog muscle at the same temperature (20° C)

In the smooth anterior byssus retractor muscle (ABRM) from *Mytilus* a latency relaxation did not occur in response to a single shock but it appeared after two shocks given at intervals of less than 10 sec When the muscle was stimulated with successive shocks at constant intervals of 2–10 sec we found that as the amount of tension held by the muscles increased from one shock to the next so did the latency relaxation The latency relaxation showed no relation to the amount of tension developed after each shock

The rate of tension drop of latency relaxation remained the same during the successive stimulation at constant intervals *i e* it was independent of amount of tension acting at the moment of stimulation With short intervals between successive shocks (*e g* 2 sec) both the rate and the amount of tension drop of the latency relaxation were higher than at 5 sec intervals At 15 sec intervals the latency relaxation was small regardless of the amounts of initial and subsequently developed tension In the ABRM the amount of contractile activity present is known to be greater after 2 sec than after 5 sec following a shock and after 15 sec very little activity can be detected (*Lowy* and *Millman* 1963)

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Lundberg A and L Vyklicky (Institute of Physiology University of Goteborg Sweden) BRAIN STEM CONTROL OF RE FLEX PATHS TO PRIMARY AFFERENTS

A large negative dorsal root potential (DRP) can be evoked in the lumbar cord on stimulation of different regions in the brain stem (*Carpenter et al* 1962) This negative DRP is caused by pri mary afferent depolarization With other parameters of stimulation (in particular low frequency) a positive DRP could be evoked from the lower brain stem It is suggested that the positive DRP is caused by a primary afferent hyperpolarization The effect is possibly due to removal of a tonic depolarization evoked by reflex action because the same stimulation of the brain stem inhibits transmis sion in spinal reflex paths to primary afferents

Carpenter D I Engberg and A Lundberg Experientia 1962 *18* 450

The time from the stimulus to the first sign of tension drop (30 msec at 20° C) was independent both of the intervals between stimulation and of the muscle length In a muscle developing a maximum tetanic tension of about 20 g the largest latency relaxation was about 3 mg

The results are interpreted on the assumption that stimulation initiates two opposing processes one causing the drop in tension (A) and another which generates positive tension (B) The amount of tension drop of the latency relaxation will thus be determined by (1) the rate of process A which is dependent upon the amount of contractile activity present in the muscle at the time of stimulation, (2) the time of onset of process B and (3) the rate of process B

Abbott B C and J M Ritchie, J Physiol (Lond) 1951 *113* 330

Abbott B C and J Lowy J Physiol (Lond) 1956 *133* 8p

Lowy J and B M Millman Phil Trans B 1963 *246* 105

Rauh F Z Biol 1922 *76* 25

Sandow A J cell comp Physiol 1944 *24* 221

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Lundgren, Ove and Ingemar Wallentin (Department of Physiology University of Goteborg Sweden) FLOW RESISTANCE CAPILLARY FILTRATION COEFFICIENT AND REGIONAL BLOOD VOLUME IN LYMPH GLANDS OF THE CAT

Circulatory changes in the mesenteric lymph glands have been investigated in the cat Changes in blood flow and capillary filtration coefficient (CFC) were studied at different levels of vascular tone induced by constant infusion of isopropyl noradrenaline and by stimulation of the regional sympathetic vasoconstrictor fibres During resting conditions blood flow ranged between 20 and 60 ml/min/100 g tissue and CFC between 0 01 and 0 05 ml/min/mm Hg/100 g tissue Infusion of isopropyl noradrenaline increased the flow to 180 ml/min/100 g tissue and CFC to about 0 10 ml/min/mm Hg/100 g tissue The maximum values for blood flow and CFC were about three times greater than those reported for skeletal muscle of the cat This implies a difference in vascularization of the two tissues The data also indicate a wide range of control of blood flow and CFC which may be related to the varying metabolic demands of the lymphatic tissue during different physiological and pathological conditions

The regional blood volume during resting conditions was greater than that of skeletal muscle indicating a richer vascularization The changes of blood volume induced by injections of different vasodilating and vasoconstricting substances and by regional sympathetic stimulation were of the same relative magnitude as those reported for skeletal muscle

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Lundgren, C and S Lichtneckert (Institute of Physiology, University of Lund Sweden) DISTRIBUTION OF INSPIRED GAS DURING VENTILATION WITHOUT RESPIRATORY MOVEMENTS

The relative importance of elastic and flow resistance for the uneven distribution of inspired gas in the lungs has so far been difficult to evaluate Lung ventilation in a ' barospirator (*Thunberg* 1924) was studied by means of the nitrogen elimination technique (*Lichtneckert* and *Lundgren* 1963) because it offers a possibility for cyclical ventilation without respiratory movements so that the elasticity component can be disregarded

Thunberg T Hyg Revy 1924 *13* 142
Lichtneckert S J A and C E G Lundgren J appl Physiol 1963 In press

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Lundquist, F, N Tygstrup and K Winkler (Department of Biochemistry and Cardiologic Laboratory of the Medical Department B Rigshospitalet University of Copenhagen Denmark)
THE INFLUENCE OF FRUCTOSE ON ETHANOL METABOLISM IN MAN

Large doses of fructose are known to increase the rate of ethanol disappearance from the circulating blood to nearly twice the value found when only ethanol is given The experimental material and procedure used in an attempt to explain the mechanism of this effect has been described (*Winkler Tygstrup* and *Lundquist* 1963 *Tygstrup Winkler* and *Lundquist* 1963) The amount of ethanol metabolized by the liver increased from 1 52 mmoles/min in experiments with ethanol alone and to 2 78 mmoles/min in experiments with ethanol plus fructose The rate of formation of free acetate by the liver was increased in proportion to the ethanol metabolism (from 1 27 to 2 48 mmoles/min) The arterial ethanol concentration was kept constant around 3 5 mM while the arterial acetate concentration rose from 0 84 mM in experiments with ethanol alone to 1 40 in experiments with ethanol plus fructose

In an attempt to explain the increase in the capacity of the liver for ethanol dehydrogenation *Holzer* has suggested two mechanisms 1 Oxidation of the alcohol dehydrogenase (ADH) – NADH complex* formed during ethanol dehydrogenation by glyceraldehyde known to be formed in the liver from fructose In this way the rate limiting step in the overall process is circumvented 2 Oxidation of NADH (formed from ethanol and NAD)* by fructose catalysed by polyol dehydrogenase These two mechanisms should result in increased levels of glycerol and sorbitol respectively in the hepatic venous blood Measurement of the relevant substances showed that sorbitol and glycerol formation could account for about 28 per cent of the increased ethanol oxidation

A third mechanism is now suggested whereby increased amounts of NAD are made available for ethanol dehydrogenation through increased oxidation of the NADH formed in the cytoplasm The mediator of this increase in NADH oxidation is glycerophosphate

NADH reduced nicotineamide adenine dinucleotide
NAD nicotineamide adenine dinucleotide

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Lundin, G and D Thomson (Institute of Physiology University of Lund, Sweden) STUDIES OF CARDIAC OUTPUT DURING INTERMITTENT AND CONTINUOUS WORK

The cardiac output was determined by a CO rebreathing method during intermittent and continuous work The total work load during an experimental period was the same in both types of work The experiments are of interest when comparing the training effects of the two types of work

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Manninen, Kimmo, Matti Maenpaa and Aimo Pekkarinen (Department of Pharmacology University of Turku Finland)
EFFECT OF DRUGS INFLUENCING THE SYMPATHETIC NERVOUS SYSTEM UPON THE BLOOD GLUCOSE REGULATION IN RATS DURING ETHER ANESTHESIA

The blood glucose content of normal rats (1 06±0 03 g/l) rose in ether anesthesia to at most 1 34±0 06 g/l in 30 min and decreased in spite of the anesthesia within 2 5 h to 1 04±0 09 g/l Administration of adrenaline (1 mg/kg) during prolonged ether anesthesia produced an increase in blood glucose to 1 72±0 10 g/l within 90 min indicating abundant glycogen stores After hydrocortisone treatment for four days (5 mg/kg/24 h) ether anesthesia caused an initial increase in blood glucose in 30 min to 1 40±0 08 g/l) persisting for 90 min (1 50±0 11 g/l) The prolonged increase in blood glucose might be due to the increase in gluconeogenesis as well as to a retardation of glucose oxidation caused by hydrocortisone The average rise in blood glucose was not as pronounced as after adrenaline administration

In rats treated for four days with large doses of reserpine (1 mg/kg/24 h) the blood glucose was above the control level and increased slowly during ether anesthesia as long as the anesthesia lasted (within 2 5 h to 1 58±0 10 g/l) Large reserpine doses increased the glucocorticoid secretion and thereby the storage of glycogen (*Bal er et al* 1962) but decreased the reserves of noradrenaline and adrenaline This may explain the slow rise in blood glucose during ether anesthesia after reserpine treatment

With drugs that (1) decrease the noradrenaline and adrenaline content (guanethidine 20 mg/kg/day α methyldopa 25 mg/kg/day) (2) inhibit the secretions of these transmitters from the sympathetic nerve endings (bretylium 25 mg/kg/day guanethidine) or (3) block their synthesis (α methyldopa) the initial increase of the blood glucose before and during ether anesthesia is somewhat less than in normal rats but was later somewhat higher than in the control group

Balzer H and D Plam Naunyn Schmiedebergs Arch exp Path Pharmak 196? *243 65*

formed from fructose *via* glyceraldehyde Glycerophosphate is known to be oxidized intramitochondrially with formation of dihydroxyacetone phosphate which in turn is reduced by NADH in the cytoplasm

This hypothesis is supported by experiments with rats in which intraperitoneal injection of fructose + ethanol gave rise to a significant increase in hepatic glycerophosphate compared to control experiments with ethanol or fructose alone These experiments also confirm the absence of glycogen formation from fructose when ethanol was given simultaneously (*Tygstrup Winkler* and *Lundquist* 1963)

Tygstrup N K Winkler and F Lundquist 1963 This volume p 153–154
Winkler K N Tygstrup and F Lundquist 1963 This volume p 167

chamber so that it was virtually divided In this case the two parts hemolyzed slowly at slightly different times or at different rates Even with such damage there was no hole as would be evidenced by hemoglobin escape within fraction of a second These results do not contradict the concept of the red cell as an internally structureless sac bounded by a thin membrane (cf *Teitel Bernard* 1932)

Ling, G N A Physical Theory of the Living State New York 1962
Marsden N and R Neihof Acta Soc Med upsalien 1957 *62* ii
Rockwood R J Lab clin Med 1924 *10* 19
Teitel Bernard A Arch roum Path exp 1932 *5* 389

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Marsden, N V B (Institute of Physiology, University of Uppsala Sweden) HEMOLYSIS INDUCED BY MICRO NEEDLES

Red blood cells held in a micro-holder in isotonic solution were impaled with micro-needles (tip diameter about 0 1 μ) The observations were made in a shallow chamber at 25° C by means of an inverted microscope with water immersed condenser and phase contrast

Hemolysis generally occurred within varying periods up to about a minute after the micro-needle had impaled the cell The cell became spherical before hemolysis Sometimes hemolysis did not occur even after several impalements (cf *Rockwood* 1924) Proof that the cell can be resistant at least after contact with a glass microelectrode was found by *Marsden* and *Nelhof* (1957), who recorded repeatedly a potential of about − 8 mV when a capillary electrode was pushed against the same cell on successive occasions

The hemolytic process took several seconds before it was complete Escape of refractile material from the region of the microneedle was never seen (although, the slowness of hemolysis might make this difficult to observe) However hemoglobin may leak at least macroscopically evenly over the cell surface If this is the case, local trauma produced by the micro needle presumably induces either a general change over the surface or some effect in discrete distal sites (cf *Ling* 1962) Removal of the micro-needle before the onset of hemolysis was without effect

The spherical ghost was difficult to dislodge from the needle but when this was successful it remained spherical If a ghost attached to a micro-needle was pushed against an unhemolyzed cell secured by the microholder, the cell was distorted the ghost remaining spherical This suggests that the ghost is more rigid than the red blood cell in agreement with the observation that hemoglobin liberation did not occur only at the micro-needle site *i e* that a ghost so produced is not a sac with a hole in its wall These ghosts can be maltreated between two micro needles resulting in small irregular clumps of debris

Attempts to produce a hole in the cell so that hemoglobin would escape with great rapidity failed even with bigger needles or when a needle was drawn across a cell lying on the floor of the

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Mellander, Stefan (Department of Physiology University of Goteborg Sweden) TRANSCAPILLARY FLUID EXCHANGE IN EXPERIMENTAL BURN AND AFTER ADMINISTRATION OF 48/80

Acute circulatory changes produced by second degree burn in the hind paw of cat were studied by quantitative methods The reactions of the resistance vessels were followed continuously (pressure—flow measurement) The rate of net transcapillary movement of fluid was measured by continuous recording of the paw volume Changes in capillary fluid permeability were followed by determination of the capillary filtration coefficient (CFC) Second degree burn was produced by exposing the paw to hot water (75° C) for 30 sec The thermic lesion produced a pronounced long lasting dilatation of the regional resistance vessels Immediately after burn there was a rapid loss of intravascular fluid to the injured tissue (oedema formation) amounting to 10 to 15 ml/min/100 g tissue With time the rate of transfer gradually declined CFC increased about 30 per cent during the phase of rapid fluid transfer indicating that capillary permeability was only little changed From these data the magnitude of the driving force required for producing the recorded net transcapillary movement of fluid could be calculated and it comprised as much as 200 to 250 mm Hg shortly after the lesion Since the changes in effective filtration and plasma colloid osmotic pressures can hardly account for more than 1/10 of this value it was assumed that extravascular osmolality was increased by some mechanism If so fluid would rapidly be lost from the circulation and since transcapillary diffusion of small molecules is much more rapid than plasma flow an approximate transcapillary osmotic equilibrium would be established rapidly Cryoscopic measurements on plasma drained from the burnt region showed an increased osmolality compared to normal amounting to 10 to 15 mOsm immediately after burn or 170 to 255 mm Hg and then a gradual decline Simultaneously there was an increase of local tissue osmotic pressure The oedema formation therefore seems to be dependent mainly upon a diffusion process due to a transcapillary osmotic pressure gradient established during burn Instead of selectively damaging the capillary membrane as is generally assumed burn

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Marsden, N V B and H R Ulfendahl (Institute of Physiology University of Uppsala Sweden) HALIDE AND PROTEIN SEPARATION BY GEL FILTRATION

In experiments on renal function using bromide and iodide it is necessary, since chloride is a normal cell constitutent, to determine individual halide concentrations when more than one halide is present Analyses of such mixtures present certain difficulties (*Öbrink* and *Ulfendahl* 1959)

It is advantageous to separate the halides before their determination which can then be made by the same method for all halides A tightly cross-linked dextran gel* (Sephadex) exhibits considerable selectivity to chloride, bromide and iodide which were separated completely in that order on a column 70 cm long (*Marsden* 1963) The peaks of chloride and iodide and bromide and iodide, were so widely separated that collection of the eluted halides was possible in single samples With bromide and chloride or with the three halides the peaks were rather close together so that serial sampling was necessary to resolve these components

The gel filtration has two additional advantages The degree of dilution especially for iodine is much less than with Dowex 1×10 for example with which the halides can be separated (*Riemann* 1954) Renal tubular fluids often contain proteins including hemoglobin under the conditions of the experiments which may interfere with halide determinations With gel filtration proteins were eluted well in advance of the first halide peak (chloride) and thus eliminated (*Flodin* 1962) This method thus combines halide separation and deproteinization in a single step

Flodin P Dextran Gels and their applications in gel filtration Uppsala 1962
Marsden N V B 1963 Unpublished observations
Öbrink K J and Maj Ulfendahl Acta Soc Med upsalien 1959 *64* 384–391
Riemann W Rec chem Progr 1954 *15* 85

* Our thanks are due to AB Pharmacia Uppsala for the synthesis of the gel

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Molbech Svend (The Danish National Association for Infantile Paralysis Tuborgvej 5 Hellerup Denmark) AVERAGE PER CENTAGE FORCE AT REPEATED MAXIMAL ISO METRIC MUSCLE CONTRACTIONS AT DIFFERENT FREQUENCIES

Maximal isometric force in four different muscle groups was determined by using strain gauge dynamometers and an automatic measuring bridge The muscle groups tested were those used for the following attempted movements 1 thumb pressure (tip of thumb against side of second finger) 2 handgrip (suitable grip size) 3 horizontal pull (elbow flexed 90 degrees) and 4 leg extension with one leg (knees slightly bent) The highest value obtained in three attempts was considered to be the maximum value

The subjects worked after a metronome at varying frequencies performing from 6 to 29 maximal isometric muscle contractions/min A total of 74 series of experiments were carried out The working time was 10 min and after 2 to 6 min the force of the contractions usually reached a steady state *i e* levelled off at an average value The individual variations in the force of the contractions were rather large and in some tests it was difficult to determine the average force even of those contractions made in the last few minutes of the working period The level chosen in each test is taken to represent the force which on an average can be mobilized over a long period (cf steady state) at the frequency in ques tion The subjects had no opportunity to see their own scores while working It was attempted to keep the motivation at a high level on all occasions

At a frequency of 6 maximal isometric contractions/min about 85 per cent of the maximal force could be mobilized When the fre quency was increased the average percentage of maximal force that could be mobilized decreased almost rectilinearly and at a fre quency of 29 contractions/min about 60 per cent of the maximal force could be produced No clear cut difference in the percentage for the different muscle groups was found although thumb pressure was somewhat lower than that of the others The standard error of force for all muscle groups at about corresponding frequencies varied from 2 66–4 43 per cent

might mainly affect the cellular membranes, permitting release of intracellular molecules which directly or after enzymatic decomposition temporarily tend to raise extravascular osmolality. To judge from preliminary experiments in which the histamine liberator 48/80 was administered it appears that also other types of rapid oedema formation (*e.g.* in allergic manifestations) might be dependent upon an increase of tissue osmolality.

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As expected the pulse rate was unstable in each test, when leg extension was excluded there was little correlation between pulse increase and increase in contraction frequency Only at higher fre quencies the pulse rate increased significantly with the size of the muscle group

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Møller, Aage (Department of Physiology II Karolinska Institutet Stockholm Sweden) ON THE TRANSMISSION CHARACTERISTIC OF THE MIDDLE EAR

The function of the middle ear of anesthetized cats was investigated The amplitude and phase angle of the vibration of the malleus the incus and the round window for constant sound pressure level at the eardrum were measured by a capacitive probe By comparing the inverse acoustic impedance at the eardrum with the velocity of the vibration of the malleus at constant sound pressure at the eardrum it was found that the eardrum acts as a rigid piston up to about 4 kcps A comparison of the amplitude of vibration of the malleus and of the round window indicates that the coupling between the malleus and the cochlear fluid is tight

A mathematical model of the transfer function from sound pressure at the eardrum to volume displacement at the round window was developed Thereby the transfer function of the middle ear could be estimated solely from the acoustic impedance at the eardrum which can be measured in human subjects

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Møller, J V (Department of Biochemistry, University of Aarhus Denmark) EXPERIMENTS ON THE RENAL HAND LING OF URATE IN THE RABBIT

In a previous study urate clearance ratios (C_{ur}/C_{cr}) greater than one were reported (*Møller* 1962) On the other hand, urine/ plasma ratios less than one were found in some experiments during infusion of lactate To investigate whether uric acid is simultane ously secreted and reabsorbed by active mechanisms in the rabbit we have studied the effect of probenecid on the renal excretion of uric acid during urate infusion In agreement with *Poulsen* (1955), we found that probenecid exerts a depressing effect on uric acid clearance, in contrast to the uricosuria encountered in man and mongrel dog In 7 experiments C_{ur}/C_{cr} fell from a mean of 1 82 to 0 88 (0 81–0 94) The fall in the clearance ratios was not in fluenced by addition of mannitol to the infusion fluid Even during excessive osmotic diuresis urate clearance did not equal the crea tinine clearance The fall in urate clearance to a value slightly below glomerular filtration is consistent with the view that at least a part of uric acid is reabsorbed by a probenecid insensitive me chanism

We have furthermore studied the secretory and reabsorptive processes in experiments in which the plasma urate concentration (P_{ur}) was varied The curves relating net secretion to plasma uric acid concentration showed an initial dip below zero – indicating net reabsorption at low plasma levels – and then rose linearly when the plasma urate concentration was raised from 15 to 175 μg/ml This would be expected if the true secretion of uric acid is proportional to P_{ur} and counteracted by an active reabsorption having a maximum capacity (T_m) On the basis of these assump tions T_m was calculated for the active reabsorption from the inter ception of the secretory limb of the graph with the Y-axis The slope of the graph is a measure of the true secretory rate From these values excretion curves can be constructed which fit the ob- served values

Møller J V Acta physiol scand 1962 54 30–36
Poulsen H Acta pharmacol (Kbh) 1955 11 277

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Neville, A C (Zoophysiological Laboratory B University of Copen hagen Denmark) DAILY GROWTH ZONES IN INSECT SKELETONS

The two main types of insect cuticle solid and rubber like cuticle (*Weis Fogh* 1960) both grow by daily increments giving rise to daily growth zones (*Neville* 1963 a b) So far skeletal growth zones have been proved to be the expression of a daily growth rhythm only in locusts and grasshoppers (*Orthoptera*) but zones of similar appearance occur in the solid cuticle of cockroaches (*Dictyoptera*) bugs (*Hemiptera Heteroptera*) frog hoppers (*Hemiptera Homoptera*) dragonflies (*Odonata*) earwigs (*Dermaptera*) and spiders (*Arachnida*)

The zonation in the locust skeleton is due to differences in diurnal and nocturnal deposits one pair of zones being deposited every 24 hours Locusts reared in double rhythm (6 hours day 6 hours night) or halved rhythm (24 hours day 24 hours night) produce both solid and rubber like cuticles with twice or half as *many* zones as control reared animals (12 hours day 12 hours night)

Since the physical and chemical properties of the two sorts of cuticle are distinct it is not surprising to find differences in chemistry structure and mode of formation of their growth layers In rubber like cuticle the zonation is due to layered variations in fluorescence of the rubber like protein resilin brightly fluorescent day zones alternate with faintly fluorescent night zones *In solid* cuticle the layering is due to the polysaccharide chitin nocturnal zones have the chitin crystallites organized into lamellae whereas diurnal zones have not

The daily zonation of resilin in rubber like *cuticle* has to be driven by a temperature rhythm a light-dark rhythm by itself proved inadequate By contrast the chitin lamellation *rhythm in* solid cuticle is driven endogenously being present in *locusts reared in constant dark* at 35 C It can only be inhibited by constant light and constant temperature (35° C) acting together being unaffected when only one factor is constant. Rubber like cuticle also contains chitin lamellae but here lamellation cannot be *inhibited* by varying external factors Similarly *chitin lamellation* in solid exocuticle which is all deposited before ecdysis cannot be inhibited yet chitin

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Møller, J V (Department of Biochemistry *University of Aarhus* Denmark) EXPERIMENTS ON THE RENAL HANDLING OF URATE IN THE RABBIT

In a previous study urate clearance ratios (C_{ur}/C_{cr}) greater than one were reported (*Møller* 1962) On the other hand, urine/plasma ratios less than one were found in some experiments during infusion of lactate To investigate whether uric acid is simultaneously secreted and reabsorbed by active mechanisms in the rabbit we have studied the effect of probenecid on the renal excretion of uric acid during urate infusion In agreement with *Poulsen* (1955), we found that probenecid exerts a depressing effect on uric acid clearance, in contrast to the uricosuria encountered in man and mongrel dog In 7 experiments C_{ur}/C_{cr} fell from a mean of 1 82 to 0 88 (0 81–0 94) The fall in the clearance ratios was not influenced by addition of mannitol to the infusion fluid Even during *excessive osmotic diuresis urate clearance did not equal the creatinine* clearance The fall in urate clearance to a value slightly below glomerular filtration is consistent with the view that at least a part of uric acid is reabsorbed by a probenecid insensitive mechanism

We have furthermore studied the secretory and reabsorptive processes in experiments in which the plasma urate concentration (P_{ur}) was varied The curves relating net secretion to plasma uric acid concentration showed an initial dip below zero – indicating net reabsorption at low plasma levels – and then rose linearly when the plasma urate concentration was raised from 15 to 175 µg/ml This would be expected if the true secretion of uric acid is proportional to P_{ur} and counteracted by an active reabsorption having a maximum capacity (T_m) On the basis of these assumptions T_m was calculated for the active reabsorption from the interception of the secretory limb of the graph with the Y axis The *slope of the graph is a measure of the true secretory rate* From these values excretion curves can be constructed which fit the observed values

Møller J V Acta physiol scand 1962 *54* 30–36
Poulsen H., Acta pharmacol (Kbh) 1955 *11* 277

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Nielsen, Klaus H (Institute of Biochemistry University of Aarhus Denmark) DETERMINATION OF AROMATIC ACIDS IN URINE FROM RATS LOADED WITH L PHENYL ALANINE AND PHENYLPYRUVIC ACID

Paper chromatographic methods have been developed for the quantitative determination of aromatic α keto acids and phenolic acids in urine The aromatic α keto acids were converted to 3 alkyl quinoxalinols by reaction with o-phenylenediamine The quinoxalinols were then separated by two dimensional paper chromatography and th quantities determined by ultraviolet spectrophotometry The phenolic acids were extracted from urine and separated by two dimensional chromatography The chromatograms were sprayed with aqueous formaldehyde and diazotized p-nitroaniline and the quantities of the azo dyes determined spectrophotometrically

The methods were applied in studies of the effect of continuous subcutaneous infusion of L phenylalanine and phenylpyruvic acid into rats The infusion produced an increased urinary excretion of the aromatic acids usually found in urine from phenylketonurics

lamellation in solid endocuticle, deposited by the same cells after ecdysis, can be inhibited

Daily growth zones are being used as a field method for finding the exact age of an insect, and as an experimental method for the comparative investigation of growth and deposition of solid cuticle and resilin, and of chitin lamellogenesis

Neville A C Daily growth layers in locust rubber like cuticle influenced by an external rhythm J Insect Physiol 1963 a *9* 177–186

Neville A C Growth and deposition of resilin and chitin in locust rubber like cuticle J Insect Physiol 1963 b In press

Weis Fogh T A rubber like protein in insect cuticle J exp Biol 1960 *37* 889–907

Abstr XI Scand Physiol Congr Copenhagen 1963
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Nilsson, B Y and C R Skoglund (Department of Physiology Karolinska Institutet Stockholm Sweden) STUDIES OF THE TACTILE HAIRS AND ADJACENT PACINIAN CORPUSCLES ON THE CAT S FORELEG

In addition to the vibrissae on the upper lip most mammalian species have scattered tactile hairs also on other parts of the body At the wrist of the cat s forelimb there is thus regularly a tuft of 5–6 vibrissae like hairs protruding from a small easily palpable elevation in the skin formed by the hair follicles each of which contains a blood sinus Contrary to earlier described tactile hairs these have a number of Pacinian corpuscles situated close to the blood sinus Since the whole structure is innervated by a separate branch of the ulnar nerve and gets its main blood supply *via* a small branch from the ulnar artery it is well suited for a functional analysis of the receptors under controlled milieu conditions

In contrast to the characteristic fast adapting discharges elicited by touching ordinary hairs in the same area bending of a tactile hair produced a very slowly adapting discharge After a transient initial discharge of more than 200 per sec a steady frequency level of 20 to 40 per sec was usually maintained for several minutes without appreciable decline continuing discharges without pronounced fatigue were recorded during observation periods of up to 10 min Certain signs of directional sensitivity were observed Some units exhibited spontaneous discharges which could not be stopped by changing the position of the hair After activation of such units a silent period occurred the length of which depended on the duration of the stimulus In these and in certain other respects the tactile hair receptors reacted like those of the vibrissae of the lips (*Fitzgerald* 1940)

Some of the units observed by recording from nerve filaments showed rapidly adapting discharges to vibratory stimuli *e g* tapping on the experimental table and these could be traced to Pacinian corpuscles by direct recording from the nerve fiber of a single corpuscle Pulse synchronous discharges often occurred as a result of the high mechanical sensitivity of these receptors the general response patterns of which were found to be similar to those described for

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Nienstedt, Walter (Department of Physiology University of Turku Finland) A RAPID METHOD FOR PLASMA PROGESTERONE DETERMINATION

A new method for plasma progesterone determination is presented that reduces the time required for an assay from 36 hours to about 4 hours (for references see *Short* 1961) The method uses a preliminary purification procedure on Florisil followed by a final assay by gas chromatography and is based on the following observations of the mobility of twenty reference steroids on Florisil

In contrast to what is reported of most liquid liquid or other liquid solid chromatography systems a ketone group often has a greater retention effect than a hydroxyl group Thus, progesterone is eluted after *e g* pregnanediol, together with polar compounds facilitating its separation from interfering non polar material Only the most non polar steroids (*e g* pregnanedione) share the property of the plasma lipids of being less strongly adsorbed when Florisil is deactivated with water (*Carroll* 1961) The polar steroids, *e g* cortisol are retained more The mobility of progesterone is not appreciably influenced The treatment of water-equilibrated Florisil with 10 per cent acetic acid (5 min) has only a slight influence on the mobility of steroids but purer fractions can be obtained when plasma lipids are chromatographed

Twenty ml of plasma is deproteinized with 60 ml of ethanol filtered through glass wool and the precipitate washed twice with 10 ml of ethanol After adding 4 ml of butanol the filtrate is evaporated under reduced pressure in a rotating evaporator The residue is washed three times with 3 to 5 ml of ethyl ether and the washings are successively applied to a column (17 5 cm × 0 3cm) packed with acid treated Florisil washed five times with acetone and twice with ether, and more ether (up to 25 ml) is added to the column The ether eluate containing most of the plasma lipids is discarded Progesterone is eluted with 7 ml of 50 per cent acetone in ether The fraction is evaporated dissolved in ether and re chromatographed on a smaller column (7 5 cm × 0 2cm) packed with water equilibrated, acetone and ether-washed Florisil After discarding 10 ml of ether, progesterone is eluted with 10 ml of 15 per cent acetone in ether

Carroll K K J Lipid Res 1961 2 135–141
Short R V., in Gray C H and A L Bacharach (ed) Hormones in ... Press London and New York 1961 p 379–437

Abstr XI Scand Physiol Congr Copenhagen 1963
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Nilsson, B Y and C R Skoglund (Department of Physiology Karolinska Institutet Stockholm Sweden) STUDIES OF THE TACTILE HAIRS AND ADJACENT PACINIAN CORPUSCLES ON THE CATS FORELEG

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Pacinian corpuscles at other loci, *e g* deeper in the cat s foreleg (*Skoglund* 1960)

The functional significance of the combination of the two different receptor types in this structure will be discussed

Fitzgerald O J Physiol (Lond) 1940 *98* 163
Skoglund C R Acta physiol scand 1960 *50* 385

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Ofstad, J (The Chr Michelsen Institute and the School of Medicine (D partment A) University of Bergen Norway) HAEMO GLOBIN CONCENTRATION IN THE HUMAN RENAL VEIN IN THE SUPINE AND IN THE TILTED (HEAD UP) POSITION AND DURING THE VALSALVA MA NOEUVRE

The increase in renal weight with increasing arterial pressure cannot be explained by the vasoconstrictor theory of the autoregula tion of the renal vascular resistance but is consistent with the theory of intrarenal vascular compression (*Hinshaw* 1961) Assuming that the weight increases not only due to an increase in the pre venous vascular bed but to increased capillary filtration as well changes in renal vascular resistance may be expected to be followed by transient variations in the haemoglobin concentration in the renal vein

Continuous recording of the oxygen saturation and of the haemo-globin concentration (light transmission at 805 mu) in the renal vein and ordinary spectrophotometric determinations were per formed The investigation comprised 127 experiments on 70 indi viduals with normal or diseased kidneys

In two out of 19 experiments oscillating haemoglobin concen tration variations of 3 per cent were observed in the tilted position spontaneous concentration changes did not occur in the supine posi tion

During the Valsalva manoeuvre an immediate repeatable tran sient and parallel reduction in the haemoglobin concentration and the oxygen saturation occurred The minimal values were reached in 13 sec the whole period of reduction lasting about 30–40 sec The maximal reductions were 40 per cent for haemoglobin and 30 per cent for the oxygen saturation Assuming that the haemoglobin concentration changes were caused by dilution of the venous blood with a haemoglobin free fluid the composition of this deluting fluid was calculated from analyses of blood sampled before and dur ing the Valsalva manoeuvre On the average the protein and chloride concentrations in the diluting fluid were 1 9 per cent and 125 mEq/1 respectively The albumin/globulin ratio the freezing point depres sion and the concentrations of sodium potassium and urea did not

differ significantly from the corresponding values in renal serum the inulin and para-amino hippurate concentrations were lower

Application of the Hamilton principle to the recorded curves showed that the renal blood flow was greatly reduced during the Valsalva manoeuvre, probably the renal interstitial fluid is transported into the circulating blood from which it is later reabsorbed The mechanism of this process remains obscure

Hinshaw L B and D M Worthen Circ Res 1961 *9* 1156

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Oro, Lars (Department of Internal Medicine Karolinska Sjukhuset and King Gustaf V Research Institute Stockholm Sweden)
THE EFFECT OF CAROTID OCCLUSION AND CENTRAL VAGUS STIMULATION ON THE PLASMA FREE FATTY ACIDS (FFA) AND THE BLOOD PRESSURE IN THE DOG

The role of the sympathetic nervous system for the regulation of the plasma free fatty acids has become evident in recent years An increased activity in the sympathetic nervous system is frequently associated with a rise in the plasma FFA concentration To study the possible role of baroreceptor reflexes for the regulation of the plasma FFA dogs were anesthetized with pentobarbital In one series of experiments the two common carotid arteries were occluded during two periods of 30 min duration 60 min apart The mean blood pressure increased markedly during the two occlusions from 127 to 197 and from 120 to 173 mm Hg respectively The mean plasma FFA concentration increased only slightly during the two periods from 0 33 to 0 46 and from 0 37 to 0 47 mEq/l respectively In a second series of experiments the two vagus nerves were cut The central ends were stimulated electrically during two periods of 30 min duration and 40 min apart (35 impulses/sec 15–20 volt duration 7 msec) Because of respiratory arrest during the stimulation the dogs were artificially ventilated during the whole experiment. The mean blood pressure increased from 110 to 163 and from 115 to 176 mm Hg during the two periods The mean plasma FFA concentration was unchanged In a third series of experiments different doses of catecholamines were infused i v for 30 min. A two- to three fold increase of the FFA concentration occurred with norepinephrine as well as with epinephrine (0 04–0 08 µg/kg/min) without any rise of the blood pressure

Thus pressor reflexes could be activated in the anesthetized dog markedly increasing the blood pressure without changing the plasma FFA concentration This suggests that the baroreceptors are of no importance for the FFA regulation The central parts of the sympathetic nervous system seem to be differentiated with respect to the regulation of the blood pressure and the FFA metabolism

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Oscarsson, O and I Rosen (*Institute of Physiology University of Lund, Sweden*) PROJECTION TO CEREBRAL CORTEX OF GROUP I AFFERENTS FROM STRETCH RECEPTORS IN FORELIMB MUSCLES OF THE CAT

It has been concluded from studies on the cat that group I muscle afferents do not project to the cerebral cortex (*Mountcastle Covian* and *Harrison* 1952) This has been confirmed with respect to hindlimb afferents by *McIntyre* (1953, 1962) However, *Amassian* and *Berlin* (1958) reported that stimulation of group I muscle afferents in contralateral forelimb nerves evoked surface positive potentials in the first somatic area

Our experiments were made on cats under deep pentobarbitone anaesthesia Stimulation of group I muscle afferents in contralateral forelimb nerves evoked surface positive potentials with a short latency in the first, but not in the second somatic area The responsible pathway was disynaptic and identified as the dorsal column medial lemniscus system The group I evoked potentials were restricted to an anterior part of the first somatic (SI) area usually considered as having a motor function Potentials evoked from cutaneous afferents appeared in two distinct parts of the SI area one posterior part corresponding to the classical somatosensory receiving area and one anterior part which coincided with that responding to stimulation of group I afferents It is suggested that the forelimb region of the SI area is differentiated into one motor area receiving a projection from group I muscle afferents and cutaneous afferents and one sensory area receiving a projection from cutaneous but not group I afferents

The group I afferents responsible for the cortical potential originated in slowly adapting muscle stretch receptors which had a low threshold for stretch and could be activated by succinylcholine Thus muscle spindle afferents with primary endings were responsible for the cortical potential though in addition there may be a cortical projection path from tendon organ afferents

Amassian V E and L Berlin Early cortical projection of Group I afferents in the forelimb muscle nerves of cat J Physiol (Lond) 1958 *143* 61P

McIntyre A K Cortical projection of afferent impulses in muscle nerves Proc Univ Otago med Sch 1953 *31* 5–6
McIntyre A K Central projection of impulses from receptors activated by muscle stretch Symposium on Muscle Receptors
Barker (editor) Hong Kong 1962 19–30
Mountcastle V B M R Covian and C R Harrison The central representation of some forms of deep sensibility Proc Ass Res Nerv Ment Diseases 1952 *30* 339–370

Ottoson, D (Department of Physiology, Karolinska Institutet Stockholm, Sweden) ACTIVITY OF MUSCLE SPINDLE AND SODIUM CONCENTRATION

The effect of changes in the external sodium concentration on the impulse discharge and the receptor potential of the muscle spindle has been investigated in isolated spindles of the frog s toe muscle (*m ext dig long IV*) Reduction of the sodium concentration of the bathing fluid was followed by a gradual fall of the spontaneous activity and a decrease of the response evoked by stretch The spontaneous activity was extinguished within 1–2 min after the sodium chloride was removed and replaced by choline chloride The impulse discharge evoked by stretch was abolished after 4 to 8 min in sodium-free solution leaving the receptor potential uncovered The effect of sodium lack on the receptor potential was studied after blocking of the impulse activity with local anaesthetics The amplitude of the receptor potential dropped to about 20 per cent of its original value in about 10 min after complete removal of the sodium of the bathing fluid Prolonged soaking (30–60 min) caused only a slight additional decline The conclusion is drawn that sodium ions play an essential role for the production of the receptor potential but that other ions are involved as well

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Oura, Erkki Kaija Konttinen and Heikki Suomalainen (Research Laboratories of the State Alcohol Monopoly Alko Helsinki Finland) THE INFLUENCE OF ALCOHOL INTAKE ON VITAMIN EXCRETION IN THE RAT

In our studies on vitamin deficiencies caused by alcohol con sumption the aim was to investigate how alcohol affects the vitamin composition of different tissues To establish the main picture, our first experimental series followed the effect of short time alcohol intake on the vitamin composition of the urine of the rat

In these experiments water was substituted by 10 per cent ethanol for about a week and then again replaced by water During these periods of alcohol intake the urine was examined for thiamine nicotinic acid methylnicotinamide pyridoxine pantothenic acid cobalamin folic acid biotin choline and an amino acid methionine On shift from water to alcohol a clear peak was observed in the daily excretion of thiamine pyridoxine and pantothenic acid When shifting back to water the excretion of these vitamins—except that of pantoth nic acid—rapidly decreased to the same level as before alcohol intake With the change from water to alcohol the excretion of nicotinic acid decreased but the excretion of methylnicotinamide increased to such an extent that the total excretion of these two vitamins rose markedly during the period of alcohol intake On the return to a water regimen the excretion of these two compounds rapidly returned to normal The biotin excretion decreased during the alcohol period and towards the end of the period the excreted amount of folic acid decreased as well For the other vitamins in vestigated for cobalamin, choline as well as for methionine no clear excretional changes could be observed

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Pekkarinen, Aimo (Department of Pharmacology, University of Turku Finland) BIOLOGICAL STANDARDIZATION OF CORTICOTROPIN ON THE BASIS OF THE CORTICOSTEROID INCREASE IN THE PLASMA OF LIVING GUINEA PIGS

Previously in the bioassay of corticotropin, the rise in the plasma corticosteroid was measured colorimetrically, several guinea pigs being used for each bioassay (*Pekkarinen* and *Tala* 1960) In the new procedure used in this study a blood sample was taken from the shoulder vein after morphine-pentobarbital medication in Fluothane® anesthesia The corticosteroid content was determined in 0 1 ml plasma samples by a modification (*Pekkarinen* and *Tala* unpublished) of the fluorometric method of *Sweat* (1954) With repeated blood sampling the basal corticosteroid content increased in male and especially in female animals Therefore, the neurogenic increase of corticosteroid after several blood samplings was prevented by intraperitoneal application of methylprednisolone (Depo-Medrol®, Upjohn Co) as depot preparation on the previous day After methylprednisolone and a large dose of methadone the sample could be taken without Fluothane® (ICI) anesthesia This procedure has the advantage that the blood sampling can be repeated with a few weeks interval The administration of methylprednisolone does not prevent the increase of corticosteroid due to corticotropin Psychopharmaca could not prevent the neurogenic increase of the corticosteroids hydroxyzine (Atarax® UBC) in large doses had only a slight inhibiting effect

The biological standardization of several different corticotropin preparations was investigated by the new corticotropin assay If the concentration of corticosteroids in plasma was less than 1 mg/l corticotropin caused a linear rise in plasma corticosteroids If the basal content of corticosteroids rose to a high level due to stress without pretreatment with methylprednisolone corticotropin no longer caused a linear increase of the corticosteroid content The most suitable rise in the corticosteroid content was usually obtained with corticotropin doses ranging from 0 015 to 0 3 I U per kg of body weight injected into the inner side of the thigh

Pekkarinen A and E Tala Abstr Int Congr Endocrinol Copenhagen 1960 p 1123

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Pekkarinen, Aimo, Mikko Linna, Enni Luostarinen and Alpo Ha kulinen (Department of Pharmacology and the Surgical Clinic University of Turku Finland) ON THE EXCRETION OF VANILMANDELIC ACID (VMA) BEFORE AND AFTER MAJOR OPERATIONS AS WELL AS DURING THE MENTAL STRESS OF EXAMINATION

After gastric and pulmonary operations the excretion of adren aline noradrenaline and total 17 hydroxycorticosteroids increased (*Halme et al* 1957 *Pekkarinen et al* 1957 *Pekkarinen* 1960) An increase in adrenaline excretion was observed also during the stress of matriculation examination (*Pekkarinen et al* 1961) The excretion into the urine of vanilmandelic acid the main metabolic product of adrenaline and noradrenaline was studied during stress using a micromodification of *Pisano*s method (*Pekkarinen* un published) The standard error of the mean for added vanilmandelic acid was 4 3 per cent in the urine and in individual series 2 4 per cent

In 75 surgical patients (subjected to 33 gastric and 10 pulmonary and heart operations) the excretion of VMA during two days before the operation was about doubled during 1 to 3 days after the opera tion After 4 to 5 days it was slightly above the preoperative values In 9 biliary operations and 6 other laparotomies the increase of VMA excretion was small In half the patients the postoperative VMA excretion was above 10 mg/24 h The most pronounced excretion (30 mg 24 h) appeared in a patient with rectal carcinoma on the second day after the operation The excretion maximum occurred more often on the second and third postoperative days than on the first day

In two groups of matriculation examination students the VMA excretion into the urine increased only occasionally The normal excretion was higher in the students than in the surgical patients before operation

Halme A A Pekkarinen and M Turunen Acta endocrinol (Kbh) 1957 *24* Suppl 32
Pekkarinen, A S Vikari and M Turunen Ann Med. exp Fenn. 1957 *35* Suppl 7
Pekkarinen A *in* The biochemical response to injury *H B Stoner* (editor) Symp of CIOMS 1960 217–268
Pekkarinen A. O Castren E Iisalo M Koivusalo A Laihinen, P E

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Pekkarinen A and E Tala Abstr Int Congr Endocrinol Copenhagen 1960 p 1123
Chem 1954 26 773

trophic hormone in which the responses of ketogenic and of total 17 OHCS are equally distinct

After two weeks of chlorpromazine treatment (100 mg 3 times daily) the average pituitary corticotrophin reserve remained almost unchanged in 10 patients during a one day metyrapone test The average excretion of the ketogenic 17 OHCS was 30 7 mg/24 h on the test day a slight decrease appeared in 5 patients After one month treatment with Sordinol® (3×10 mg) and Trilafon® (3×8 mg) the average steroid excretions were about the same during a two-day metyrapone test as when the neuroleptic drugs were given alone The mean excretion of the ketogenic 17 OHCS rose from 21 4 to 30 7 mg/24 h during the first day to 51 1 mg/24 h on the second day and to 52 1 mg/24 h on the following day In a small group of females steroid excretion decreased Old patients had pituitary corticotrophin reserves as large as the younger ones The excretion of the ketogenic 17 OHCS rose to at most 154 mg/24 h and was above 100 mg/24 h in 4 patients In 5 patients there was a small rise in excretion of the three groups of steroids indicating a low metyrapone reserve

Side effects (vomiting or dizziness) appeared in 11 patients The metyrapone treatment was therefore not repeated in 3 patients in the first metyrapone test the rise of the steroid excretion was small in two pronounced in one

Appleby J I G Gibson J K Norymberski and R D Stubbs Biochem J *1955* *60* *453*

Halme A A Pekkarinen and M Turunen, Acta endocrinol (Kbh) 1957 *24* suppl *32*

Jenkins D P H Forsham J C Laidlaw W Reddy and G W Thorn Amer J Med 1955 *18* 1

Porter C C and R H Silber Biochem J 1950 *185* 201

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Pekkarinen, A , U Rinne, H Saarimaa and C Sourander (Department of Pharmacology University of Turku, and the Psychiatric Hospital of Turku Finland) THE DETERMINATION OF THE PITUITARY CORTICOTROPHIN RESERVE BY A TWO DAY METYRAPONE (METOPIRONE®) TEST IN PSYCHIATRIC PATIENTS

Metyrapone (4 5 g/24 h, one dose every 4th hour) was given perorally to 35 patients with chronic schizophrenia 10 patients received the drug for one day, and 25 patients for two days Treatment with psychotrophic drugs was discontinued 2 weeks earlier Ketogenic 17-hydroxycorticosteroids (OHCS) were determined by a modification of the method of *Appleby et al* (1955), total 17 OHCS according to the method of *Porter* and *Silber* (1950) as modified by *Jenkins et al* (1955) and *Halme et al* (1957), and 17-ketosteroids by a modification of the methods of *Zimmermann* and of *Callow* (for references see *Halme et al* 1957)

In the one-day test the average excretion of ketogenic 17-OHCS rose from 24 1 mg/24 h to 36 2 mg/24 h, and on the following day the excretion decreased to 32 3 mg/24 h Correspondingly the average excretion of total 17 OHCS increased from 6 0 to 10 3 mg/24 h and decreased to 9 8 mg/24 h on the following day The excretion of 17-ketosteroids rose from 10 4 to 12 3 and 13 1 mg/24 h respectively

In the two-day test the average excretion of ketogenic 17 OHCS rose from 28 5 mg/24 h to 36 1 mg/24 h on the first day to 57 7 mg/24 h on the second day, and to 53 2 mg/24 h on the third day The average excretion of total 17-OHCS rose correspondingly from 8 7 to 11 6 15 2 and 12 5 mg/24 h, and that of 17 ketosteroids from 11 1 to 13 6, 20 2 and 17 4 mg/24 h

The pituitary corticotrophin reserve of psychiatric patients appears to be within limits of normal On the second metyrapone-day and the following day the level of these steroids was about twice normal the increase appearing most clearly in the excretion of ketogenic 17 OHCS The rise in total 17 OHCS was less pronounced This indicates that other steroid metabolites are excreted into the urine during a metyrapone test than during a test with adrenocortico-

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Raussi, M and K Hartiala (Department of Physiology University of Turku Finland) THE EFFECT OF SALICYLAMIDE ON THE FORMATION OF SALICYLAMIDE GLUCURONIDE IN DOGS

Eugenol is a mucigogue and desquamatory agent in the stomach Since the synthesis of neutral mucopolysaccharides (mucus) partly depends on the same energy and enzyme systems as the glucuronide conjugation process the effect of eugenol on the conjugation of a glucuronogenic substance salicylamide was studied

Fifty mg/kg salicylamide was given to 12 female dogs by intragastric feeding and the levels of salicylamide glucuronide and free salicylamide in plasma were determined After 7 days the dogs were fed the same dose of salicylamide together with a 200 mg/kg dose of eugenol in a water emulsion One hour after the feeding the level of salicylamide glucuronide in plasma was 11 ± 6 mg/l without eugenol and 4 ± 4 mg/l with eugenol The difference is statistically significant ($P < 0\ 005$) There was no difference in the levels of free salicylamide in plasma after 1 h

Thus eugenol has an inhibiting effect on the conjugation of salicylamide in the stomach Earlier studies (*Ball Hartiala* and *Pulkkinen* unpublished) have shown that eugenol inhibits the action of glucuronyltransferase

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Pulkkinen, Martti O (Department of Physiology, University of Turku, Finland) HORMONE SULPHATASE RELATIONSHIPS AND SULPHATE CONJUGATION IN THE FOETUS AND PLACENTA

The activities of arylsulphatase (I and II) and steroidsulphatase in the liver and kidneys of rats and mice were determined by a modification of the method of *Roy* (1957) Sulphate conjugation was studied by a slight modification of the method of *Nose* and *Lipman* (1959) A fraction containing the enzymes necessary for sulphate conjugation was precipitated by ammonium sulphate (1 5–2 3 M) from a high speed supernatant of homogenates of foetal liver, kidney and placenta from human and rat

The anabolic steroid, methandrostenolone, and testosterone increased the level of arylsulphatase type II, especially in the kidney The arylsulphatase level in the kidney was decreased by castration, and the gonadotrophins of human placenta or pituitary gland did not affect the level Tyrotoxine increased the activity of renal arylsulphatase type II Oestradiol decreased the level of sulphatase type II in the kidney but increased the levels of type I in both liver and kidney The level of steroidsulphatase in the liver was slightly decreased by oestradiol and methandrostenolone

Human rat and guinea-pig placentas did not conjugate p nitrophenol, dehydroepiandrosterone or oestrone with sulphate whereas human foetal liver kidney, suprarenal gland, lung and intestines were able to accomplish this conjugation in that order of decreasing effectiveness

The rat foetus could conjugate sulphate only toward the end of the gestation period The sulphate conjugation in newborn was weaker than in adult rats when related to the tissue protein level (biuret method) and wet weight Pregnancy does not produce significant changes in this conjugation in rat liver Of 12 steroids examined dehydroepiandrosterone underwent conjugation at the highest rate also in the human foetus

Nose Y and F Lipman Separation of Steroid Sulfokinases J biol Chem 1959 *233* 1348–1351
Roy A B The Sulfatase of ox liver 6 Steroid Sulphatase Biochem J 1957 *66* 700–703

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Raussi M and K Hartiala (Department of Physiology University of Turku Finland) THE EFFECT OF SALICYLAMIDE ON THE FORMATION OF SALICYLAMIDE GLUCURONIDE IN DOGS

Eugenol is a mucigogue and desquamatory agent in the stomach Since the synthesis of neutral mucopolysaccharides (mucus) partly depends on the same energy and enzyme systems as the glucuronide conjugation process the effect of eugenol on the conjugation of a glucuronogenic substance salicylamide was studied

Fifty mg/kg salicylamide was given to 12 female dogs by intragastric feeding and the levels of salicylamide glucuronide and free salicylamide in plasma were determined After 7 days the dogs were fed the same dose of salicylamide together with a 200 mg/kg dose of eugenol in a water emulsion One hour after the feeding the level of salicylamide glucuronide in plasma was 11 ± 6 mg/l without eugenol and 4 ± 4 mg/l with eugenol The difference is statistically significant ($P < 0\ 005$) There was no difference in the levels of free salicylamide in plasma after 1 h

Thus eugenol has an inhibiting effect on the conjugation of salicylamide in the stomach Earlier studies (*Ball Hartiala* and *Pulkkinen* unpublished) have shown that eugenol inhibits the action of glucuronyltransferase

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Refsum, Harald, E (The University Institute for Respiratory Physiology, Dept III, Ulleval Hospital, Oslo, Norway) *THE PLASMA pCO - pH - HCO_3^- RELATIONSHIP AT INCREASED pCO AND DECREASED pO*

To determine the average extent of the renal compensation for chronic CO retention at various levels of CO tension, the arterial O and CO tension (pO , pCO), pH and plasma HCO_3^- concentration were determined in 240 patients with pulmonary insufficiency, all breathing air pCO ranged from 50 to 95 mm Hg, and pO from 65 to 20 mm Hg When the data were grouped according to increasing pCO_2 the pH decreased progressively, but average values below the lower limit of normal were not met with until pCO_2 exceeded 70 mm Hg Correspondingly, in the pCO range of 50–70 mm Hg the increase in plasma HCO_3^- concentration with increasing pCO exceeded that observed in blood equilibrated *in vitro* with different CO tensions At pCO above 70 mm Hg the increase in HCO_3^- concentration with increasing pCO corresponded to that of *in vitro* equilibrations

The failure of the preservation of normal pH at pCO above 70 mm Hg was influenced only to a small degree by the hypoxemia The average pCO —pH—HCO_3^- relationship in patients with pCO above 80 mm Hg was similar to that found in dogs during prolonged exposure to a 12 per cent CO , 20 per cent O atmosphere (*Van Ypersele de Strihou Gulyassy* and *Schwartz* 1962)

Van Ypersele de Strihou C P F Gulyassy and W B Schwartz Effects of chronic hypercapnia on electrolyte and acid base equilibrium III Characteristics of the adaptive and recovery process as evaluated by provision of alkali J clin Invest 1962 *41* 2246–2253

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Reite, O B (Institute for Experimental Medical Research Ullevål Hospital Oslo Norway) BLOOD VESSEL RESPONSES TO ADRENALINE AND NORADRENALINE IN TISSUES PREVIOUSLY EXPOSED TO COLD

Rabbit ears subjected to two different types of cold exposure cold adaptation and frostbite were tested for vascular reactivity to adrenaline and noradrenaline Some rabbits were housed in out door cages for several weeks during the winter with one ear covered while the other ear was unprotected against the cold Other animals were kept indoors and had one ear experimentally frostbitten in a freezing bath at 10° C Changes in local blood flow following in travascular injections of adrenaline or noradrenaline were estimated by means of polarographic oxygen electrodes With one electrode in each ear the unaffected ear served as control

In cold adapted animals the blood vessels of the cold exposed ear showed a marked decrease in its response to adrenaline and noradrenaline as compared to the ear which had been protected The degree of vasoconstriction as well as its duration were significantly reduced

Frostbite produced an inflammatory reaction The blood vessels of the frostbitten ear responded to adrenaline and noradrenaline as did the vessels of the control ear however vasoconstriction in the affected ear always disappeared more rapidly than in the control

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Relander, Arvo and Carl Erik Raiha (Pediatric Clinics University Hospital, Helsinki, Finland) HEXOSES IN THE ERYTHRO CYTE STROMA

A method has been developed to estimate galactose, mannose and glucose in the erythrocyte stroma

The amount of glucose found in the untreated erythrocytes was always less than the sum of that found in washed erythrocytes and the NaCl solution used for washing This was followed by a decrease in the protein-bound hexoses in the erythrocyte stroma When erythrocytes were incubated in Ringer solution containing in creasing amounts of glucose, the increase in the hexose content of the stroma was greatest when the transport of glucose across the erythrocyte membrane was greatest The results using inhibitors of glucose transport will be discussed

Erythrocytes from patients subjected to insulin shock showed an increase in stroma bound hexoses Glucagon administration had an opposite effect on the erythrocytes During glucose tolerance tests the amount of stroma hexoses increased considerably this increase being greatest in the galactose fraction In cord blood the amount of stroma hexoses was about half that in adult blood

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Rerup, Claus and Pavo Hedner (Department of Pharmacology University of Lund Sweden) THE ASSAY OF CORTICOTROPHIN IN MICE

The assay of corticotrophin could be performed in mice by means of small sample analysis of free plasma corticosteroids Hypophysectomy was replaced by dexamethasone pretreatment The response was measured preferably in single mice weighing 20 g or more When mice of lower body weight were used the plasma of two randomly assigned mice was pooled Corticosteroids (mainly corticosterone) were determined fluorometrically in 0 25 (0 20) ml samples of plasma from heparinized blood Valid corticotrophin assays could be performed in mice both by the intravenous and subcutaneous route Compared with the adrenal ascorbic acid depletion method or the plasma corticosteroid method in the rat the assay in mice was at least five times more sensitive Forty micro-units of corticotrophin were consistently detectable Precision was dependent upon the route of administration the mean index of precision being 0 20 in the intravenous and 0 12 in the subcutaneous assay The difference was due to a steeper slope of the log-dose response line after subcutaneous administration

Contrary to the findings in the rat corticotrophin A (purified oxycel) did not differ significantly in potency estimated from subcutaneous and intravenous assays in mice when compared with crude corticotrophin (U S P corticotrophin reference standard) Accordingly results of subcutaneous assays of corticotrophin A samples in terms of the U S P standard were lower in mice than in rats The use of gelatine instead of saline as diluent in subcutaneous assays yielded slightly but not significantly higher potency estimates (25 per cent)

For intravenous corticotrophin assays the mouse method is comparable to the rat assay For subcutaneous pharmacopoeial corticotrophin assays the mouse method is unsuitable if crude corticotrophin (U S P standard) is the basis of comparison but if corticotrophin A (purified oxycel) is the standard of reference (*e g* the Third International Standard for Corticotrophin) the mouse method may be used legitimately for potency determinations of corticotrophin A samples

The advantages of the

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Richter, A W (Research Division, AB Pharmacia, Uppsala, Sweden) ERYTHROCYTE AGGREGATION, LETHAL DOSE, AND MOLECULAR STRUCTURE OF HYDROPHILIC COLLOIDS

Two hydrophilic colloids, Cellosize® (hydroxyethylcellulose) and Ficoll® (a sucrose polymer), provided additional evidence regarding the influence of molecular size and structure on erythrocyte aggregation Because of their physico chemical properties, the two colloids represent extreme examples of the influence of both highly asymmetrical and highly symmetrical molecules on the erythrocyte sedimentation rate (ESR) The *in vitro* erythrocyte aggregating effect was determined and expressed as critical concentration, using *Thorsen* and *Hints* method Fibrinogen, the most powerful ESR increasing plasma colloid with highly asymmetrical molecules and a molecular weight of 400,000, has a critical concentration of 0 2–0 3 per cent, the critical concentration of Cellosize® of a molecular weight of 250,000 was about 0 01 per cent This strong erythrocyte aggregating effect is probably due to the highly asymmetrical and non-flexible Cellosize® molecules The critical concentration of a fraction of Ficoll® with highly symmetrical molecules and a molecular weight of 2 000 000 was about 0 4 per cent Thus increased ESR may be produced clinically by the presence in plasma of low concentrations of highly asymmetrical and rigid molecules

It was investigated whether the *in vitro* erythrocyte aggregating power of colloids was correlated to their lethal dose in mice By a constant infusion technique the i v mean lethal dose was estimated for a series of hydrophilic colloids including dextran fractions, Cellosize® fractions fibrinogen and thrombin Thrombin was included as a reference compound because it is a powerful intravascular erythrocyte aggregating agent The lethal dose of other erythrocyte aggregating colloids were expressed relative to that of thrombin Relative toxicities of 1–10 per cent of that of thrombin were found for five of the most toxic compounds which also proved to possess the most powerful *in vitro* aggregating effect on human erythrocytes The concentration of five of the strongest aggregating colloid fractions necessary to produce acute lethality in mice was roughly 100 times greater than the critical

concentration required for *in vitro* ESR increasing effect and about 5 times greater than that necessary to produce maximal *in vitro* ESR increase After infusion of half the lethal dose in mice a typical syndrome was observed comprising loss of righting reflexes and resembling general anesthesia This may be due to cerebral hypoxia following severe intravascular erythrocyte aggregation The fact that the lethal dose of non-erythrocyte aggregating colloid fractions is much higher than that of aggregating colloids suggests that excessive intravascular erythrocyte aggregation may contribute to the toxicity of such colloids However other factors such as increased viscosity interference with coagulation mechanisms etc make it difficult to assess the toxic effect of severe erythrocyte aggregation *per se*

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Roos, B E, N E Anden and B Werdinius (Department of Pharmacology, University of Goteborg Sweden) EFFECT OF DRUGS ON THE LEVEL OF INDOLE AND PHENOLIC ACIDS IN THE CENTRAL NERVOUS SYSTEM

A method for determining 5 hydroxy indole acetic acid (5 HIAA) in brain has been described recently In the absence of any direct method for measuring free 5 hydroxytryptamine (5 HT) we have studied with this method the effect of reserpine and other drugs on the level of 5 HIAA in rabbit brain Six hours after injection of reserpine the value af 5-HT decreased to about 1 per cent of normal and the corresponding value of 5 HIAA rose to about 90 per cent above normal This high level was still found 24 h after the injection After 36 h the level of 5-HIAA was again normal Using a modification of this method the content of 3 4 dihydroxyphenylacetic acid (Dopac) was determined in the corpus striatum of the rabbit Two hours after a single injection of reserpine the Dopac level was at least twice normal after 12 h the level again had reached normal values Three hours after injection of α methyl DOPA into rabbits both 5 HT and 5-HIAA decreased to about 50 per cent of normal suggesting inhibition of 5 HT synthesis Simultaneous determination of the amine and its acid oxidation product might be of value in the study of the metabolism of both catechol and indole amines

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Rosenfalck, Annelise and Fritz Buchthal (Institute of Neurophysiology University of Copenhagen Denmark) ACTION POTENTIALS FROM SENSORY NERVE

The small action potentials from sensory nerve have been recorded by superposition in humans (*Dawson* 1956 *Gilliatt* and *Sears* 1958) The aim of our study was to develop a technique which would allow the small action potentials to be discriminated from noise even at a large distance from the point of stimulation and to measure conduction velocity

The digital nerves were stimulated with supramaximal pulses (12–15 mA threshold 3–5 mA) The action potentials were led off with insulated needle electrodes bared 1–3 mm at the tip and inserted close to the median nerve at the wrist, the elbow and near the axilla and recorded with single sweeps The indifferent electrodes were placed at a transverse distance of at least 25 mm from each active electrode By means of an input transformer (*Nightingale* 1958) the signal to noise ratio was improved 6 5 times (frequency range 15–5000 cps)

The amplitude of the action potential depends on the distance between the electrode and the nerve This distance was determined from the plot of amplitude as a function of depth of insertion With stimulation of the thumb the amplitude recorded unipolarly 2–3 mm from the median nerve at wrist, elbow and axilla averaged 31 μV (18–50 μV) 9 5 μV (4–17 μV) and 3 7 V(1 5–6 V) (10 subjects) by simultaneous stimulation of fingers I II and III with a delay of the stimulus to finger I to give maximum response the amplitude was increased to 51 μV (26–88 μV) 18 μV (7 5–29 μV) and 9 μV (3–12 V) respectively (6 subjects)

The conduction time was measured to the intersection of the positive negative deflection of the action potential with the base line The conduction velocity averaged 63 5±0 9 m/sec (10 subjects) and was identical between wrist and elbow and elbow and axilla. Additional peaks in the action potential recorded at the elbow indicated the presence of fibres with 10–30 per cent lower velocities

The conduction time to the wrist corresponded to a 15–30 per cent slower conduction velocity than

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Roos, B E, N E Anden and B Werdinius (Department of Pharmacology University of Goteborg Sweden) EFFECT OF DRUGS ON THE LEVEL OF INDOLE AND PHENOLIC ACIDS IN THE CENTRAL NERVOUS SYSTEM

A method for determining 5-hydroxy indole acetic acid (5 HIAA) in brain has been described recently In the absence of any direct method for measuring free 5 hydroxytryptamine (5 HT) we have studied with this method the effect of reserpine and other drugs on the level of 5 HIAA in rabbit brain Six hours after injection of reserpine the value af 5 HT decreased to about 1 per cent of normal and the corresponding value of 5 HIAA rose to about 90 per cent above normal This high level was still found 24 h after the injection After 36 h the level of 5 HIAA was again normal Using a modification of this method the content of 3 4 dihydroxyphenylacetic acid (Dopac) was determined in the corpus striatum of the rabbit Two hours after a single injection of reserpine the Dopac level was at least twice normal, after 12 h the level again had reached normal values Three hours after injection of α methyl DOPA into rabbits both 5-HT and 5 HIAA decreased to about 50 per cent of normal, suggesting inhibition of 5 HT synthesis Simultaneous determination of the amine and its acid oxidation product might be of value in the study of the metabolism of both catechol and indole amines

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Raiha Niels and Rolf Nyman (Research Laboratories of the State Alcohol Monopoly Alko Helsinki Finland) EFFECT OF ETHANOL AND NEUROGENIC STRESS ON THE ACTIVITY OF HYPOTHALAMIC CENTERS IN THE RAT

Rothballer (1953) *Mirsky et al* (1954 a b) and *Saffran* (1959) have shown close interrelations between corticotrophin stimulating factors and neurohypophysial antidiuretic factors We have examined the effect of neurogenic stimuli on the activity of the neurosecretory ganglion cells of the hypothalamus known to be activated by osmotic stimuli (*Eichner* 1952) The effect of ethanol intoxication in combination with neurogenic stress was studied as well Changes in the activity of the ganglion cells were measured as change in the volume of the cell nucleus (*Eichner* 1952) Neurogenic stress decreased the gain in weight of the growing rats It produced a hypertrophy of the adrenal gland presumably by a central activation of corticotrophin release The activity of the cells in the supraoptic and paraventricular nuclei which were easily activated by osmotic stimuli showed no response to the neurogenic stimuli Ethanol which has an inhibitory effect on changes in the ganglion cells of this area produced by osmotic tress (*Raiha* 1960) and which because of its central depressing action might affect also the stress reaction produced by neurogenic stimuli had no effect on adrenal hypertrophy produced by neurogenic stress

The results obtained are similar to those found by *Rinne* (1960) and suggest that the release of corticotrophin under the influence of neurogenic stimuli takes place without the changes in the cells of the hypothalamo-neurohypophysial system which indicate increased activity

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axilla The slowing was not due solely to distal displacement of the point of stimulation and was localized mainly in the fingers, since the velocity in the hand was of the same order as in the arm

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Salmi, Heikki A and Ilmari Lindgren (Department of Pathological Anatomy and Department of Physiology, University of Turku Finland) THE LOCALIZATION OF RADIOVITAMIN B_{12} ^{5}Co IN RAT KIDNEY

In a recent study of the metabolism of vitamin B_{1} in the foetal organism (*Salmi* 1963) it was observed by autoradiography that radiovitamin B_{12} had a characteristic localization in the proximal convoluted tubuli of rat kidney We have investigated whether this occurs only in the foetal kidney or also in the adult renal tissue

Adult rats were injected with 1 0 μC vitamin B_{1} ^{5}Co (specific activity 81 μC/μg) and the rats sacrificed at different intervals up to 4 weeks Scintillation counting was performed on kidney homogenates Samples were taken for high resolution autoradiography and ordinary stripping film technique was employed

The radioactivity increased during the first hours after injection Then it decreased continuously in agreement with results obtained from foetal kidney The autoradiography showed no differences between the adult and foetal kidney The activity was localized in the vicinity of the glomeruli and in the proximal convoluted tubuli As the activity was localized in fairly large zones near the glomeruli it also covered the juxtaglomerular cells The areas of high activity in the proximal tubuli diminished with time and after 4 weeks there was only uniform activity throughout The distal convoluted tubuli did not show any pronounced activity

The localization of radiovitamin in the proximal tubuli is of interest in view of the relationship between proximal tubular cells and vitamin B_{1} metabolism (*Jones et al* 1955 *Newberne* and *O Dell* 1959)

Jones, C C S O Brown L R Richardson and J G Sinclair Tissue abnormalities in newborn rats from vitamin B_{12} deficient mothers Proc Soc exp Biol (N Y) 1955 *90* 135–140

Newberne P M and B L O'Dell Pathology of vitamin B_{1} deficiency in infant rats J Nutr 1959 *68* 343–358

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Saltin B and S Kozlowski (Department of Physiology Gymnastiska Centralinstitutet and Department of Endocrinology Karolinska Sjukhuset, Stockholm Sweden) EFFECT OF DEHYDRATION ON THE BODY FLUIDS

Six healthy men were studied under normal conditions and after dehydration caused by sweating produced (i) in a sauna at 70–90° C (ii) by hard muscular work at 17 5–20 0° C and (iii) by light work at 36–40° C The dehydration periods lasted for 2–4 h Body weight Evan s blue space and thiosulfate space were determined before and about 90 min after the dehydration period

After the work period in the hot environment (iii) Evan s blue space decreased to the same extent as previously shown by *Adolph* (1947) but after the stay in the sauna (i) the Evan s blue space and thiosulfate space were reduced more After the hard work (ii) little or no decrease was found in thiosulfate space and Evan s blue space in spite of a water deficit of about 6 per cent of the body weight.

Water liberated from combustion of fat and carbohydrates plus water previously stored with glycogen can account for up to about 2 l of the water loss

An increase in blood potassium during and after the exercise suggests a further water leakage from the cells

Adolph, E F (editor) Physiology of Man in the Desert, Interscience New York 1947 Chapter X

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Saltin, B (Department of Physiology Gymnastiska Centralinstitu tet, *Stockholm, Sweden*) CIRCULATORY RESPONSE TO STANDARD EXERCISE BEFORE AND AFTER EX POSURE TO HEAVY EXERCISE OR HEAT

In the morning before heat exposure in a sauna oxygen uptake, heart rate, cardiac output (acetylene method) and blood lactic acid were measured on subjects working with two standard loads demanding an oxygen uptake of about 45 and 70 per cent respectively of the subject's maximum

I After the dehydration period the subjects rested until rectal temperature had decreased to resting value (37 2° C) The same measurements were repeated with the standard loads and a maxi mal load

II In another study 5 subjects were examined during work for three hours with a load corresponding to about 70 per cent of their maximal aerobic capacity After 60–90 min rest the measurements were repeated with both the submaximal load and a maximal one Cardiac output was determined by a dye dilution technique By comparing the data obtained in studies after work or heat exposure with the normal' values no difference in oxygen uptake was found with submaximal loads With maximal loads the decrease in maxi mal oxygen uptake was insignificant Heart rate was increased with the submaximal loads, most pronounced in series II Maximal heart rate was unchanged Cardiac output did not change signifi cantly either on submaximal or on maximal loads Hence the stroke volume was diminished during submaximal loads but unchanged during maximal exercise Work time and blood lactate level in the maximal exercise test were significantly reduced in both series

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Sedvall, Goran (Department of Pharmacology Karolinska Institutet Stockholm Sweden) NORADRENALINE CONTENT OF CAT SKELETAL MUSCLE FOLLOWING PRE AND POSTGANGLIONIC SYMPATHETIC DENERVATION

Noradrenaline levels in extracts of cat skeletal muscle purified by cation exchange chromatography were determined fluorimetrically Removal of the lumbosacral sympathetic chain caused a slow increase in noradrenaline content of the muscles in the hind limbs during the first two days Then muscle noradrenaline decreased abruptly and was depressed considerably after four days Fourteen days after sympathectomy the muscles were practically devoid of noradrenaline indicating that skeletal muscle noradrenaline is stored predominantly in sympathetic nerves Transection of the sympathetic chain at the level of L4 and L5 (preganglionic denervation) caused an increase in muscle noradrenaline during the first five days the average content being about 30 per cent above the normal level. Fourteen days after preganglionic denervation control and denervated muscles had about the same amount of noradrenaline The raised noradrenaline levels in the muscles during the first days after sympathetic denervations indicate that the transmitter store of the sympathetic nerves in muscle normally is in a state of slight functional depletion

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Sandberg, Nils (Department of Physiology University of Lund Sweden) SOME ASPECTS OF THE EFFECT OF CORTISONE ON WOUND HEALING

The time relationship between administration of cortisone and healing of wounds in rats and the effect of cortisone on the rate of histamine formation in skin and skin wounds were studied

Cortisone (11-dehydro-17 hydroxycorticosterone acetate) was given in water suspension intramuscularly in a daily single dose of 40 mg/kg body weight The tensile strength of healing skin wounds was reduced on the 7th day, when cortisone was given during the healing period with the first injection on the day of wound infliction or one day later When the wounds were allowed to heal for the first 2 days or longer without administration of cortisone to the rats, the tensile strength was not reduced in 7 day old skin wounds Treatment for 3 days with cortisone before infliction of wounds reduced the tensile strength of 5 and 7 day old skin wounds Pre treatment with cortisone decreased the concentration and total amount of the collagen constituent hydroxyproline in granulation tissue of polyvinyl sponges subcutaneously implanted for 5 and 7 days Thus cortisone exerts its effects during the very early course of healing

The rate of histamine formation was determined by isotope dilution in 24 and 48 h old wound tissue consisting of excised dorsal wounds in rat skin Cortisone given for 3 days before infliction of the wounds reduced the elevated rate of histamine formation in wound tissue as compared with wound tissue from the same rat before treatment with cortisone The rate of histamine formation of intact skin decreased after cortisone treatment The results suggest that the effect of cortisone on wound healing is due, at least in part to the reduced rate of histamine formation

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Skadhauge, E (Institute of Medical Physiology University of Copenhagen Denmark) DOES ANTIDIURETIC HORMONE ACT ON KIDNEY CELLS FROM THE BLOOD SIDE OR FROM THE URINE SIDE?

To investigate whether the antidiuretic hormone acts on the distal part of the nephron when reaching it from the blood or the urine side synthetic arginine vasotocin was injected into the one renal portal circulation of conscious hens lightly sedated with phenobarbital The fowls were hydrated intravenously with a one third isotonic NaCl $NaHCO_3$-glucose solution which resulted in a control urine flow of 0 6–0 8 ml/min from each ureter with a pH of 7–8 and without glucose The urine was collected in a time controlled fraction collector through small polyethylene funnels sewn over the ureters with the animals in their normal sitting position To avoid influence of the peristaltic movements collection periods were 5 min or more

A greater antidiuresis (*i e* decrease in diuresis and increase in urine osmolality) was found on the side where the injection was performed indicating that antidiuretic hormone acts when delivered to the tubular cells from the blood side

Five to ten ng of arginine vasotocin caused an antidiuresis identical with or lower than that caused by the pain involved in venipunctures With such doses no changes of the glomerular filtration rate or the renal blood flow occurred as determined by the inulin and p-aminohippuric acid clearance Higher doses however depressed both filtration rate and blood flow It was confirmed that the glomerular filtration rate increased with increasing hydration

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Smith, R S (Nobel Institute for Neurophysiology, Karolinska Institutet, Stockholm, Sweden) INTRAFUSAL MUSCULATURE IN XENOPUS LAEVIS

The electrical activity of the intrafusal musculature in *Xenopus laevis* was investigated in semi isolated muscle spindle preparations (*Koketsu* and *Nishi* 1957) by the technique of recording across the spindle capsule with a microelectrode (*Buchthal* and *Jahn* 1957) Movement of the intrafusal muscles was observed microscopically

Examination of the electrode geometry showed that the membrane current integrated over some length of fibre would be recorded The capsular membrane had a resistivity of about 400Ω cm and a characteristic length of about 0 7 mm

Threshold stimulation of the nerve was associated with a twitch like movement of the intrafusal muscle bundle and a triphasic, short latency, electrical response Supramaximal stimulation of the nerve with the large motor fibres anodally blocked was associated with a markedly slower movement of the intrafusal muscle bundle and a long latency triphasic electrical response of smaller amplitude than the short latency response Conduction velocities of the nerves giving a short latency muscle response were estimated at 10–27 m/sec and for those giving the long latency response 1–5 m/sec The amplitudes and latencies of the electrical responses were not altered when both types were allowed to occur following a single supramaximal stimulus

A single supramaximal nerve stimulus produced either three or two electrical responses of muscle two of the short latency type and one long latency, or one of each type Histological examination of the muscle spindles revealed that they contained either three (diameters approximately 15μ 15μ and 7μ) or two (diameters approximately 15μ and 7) muscle fibres

It is concluded that the muscle spindles of *Xenopus* contain large twitch and small slowly contracting muscle fibres with separate innervation from large and small diameter motor axons

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Svahn, David*, G Kahlson, Elsa Rosengren and R Thunberg (Institute of Physiology University of Lund Sweden) MOBILIZATION AND INCREASED RATE OF FORMATION OF HISTAMINE IN THE GASTRIC MUCOSA ELICITED BY GASTRIN AND FOOD

Histidine decarboxylase has been found in the gastric mucosa of all species investigated in this laboratory man dog cat rat guinea pig mouse and frog The histamine forming capacity (HFC) is much higher in the regions containing HCl secreting cells than in the pyloric region A correlation between parietal cells and HFC has been seen in horizontal frozen sections of human mucosa

On injecting gastrin in a fasted rat a mobilization of pre formed mucosal histamine ensued as shown by a reduction in the mucosal content of histamine Feeding or injecting gastrin in fasted animals evoked a striking elevation of the mucosal HFC as determined isotopically This effect has been seen in the three species so far investigated rat mouse and frog Injections of histamine had no such effect If on feeding olive oil was added to the standard meal the elevation of HFC was of lesser magnitude than without oil

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Strøier Rasmussen, P (Biochemical Institute, University of Aarhus, Denmark)* CHROMATOGRAPHIC FRACTIONATION OF PROTAMINE ON AMBERLITE IRC 50 COLUMNS WITH GUANIDINIUM CHLORIDE

In the studies to be reported protamine was fractionated chromatographically by the technique previously applied to histone (for references see, *Strøier Rasmussen Murray* and *Murray Luck* 1962)

By chromatography clupeine from herring was fractionated into two main fractions (I and II) The shape of fraction I indicates that it is heterogeneous The ascending and descending parts of the two eluate peaks were each desalted on Sephadex G 25 columns and the protamine fractions were lyophilized

Analysis of the amino acid composition of the two parts of clupeine fraction I likewise indicated heterogeneity The amino acid composition of the ascending and descending parts of clupeine fraction II were identical Calculated with respect to the smallest number of whole amino acid molecules, clupeine fraction II contains 20 (20 3) arginine, 2 (2 2) serine 2 (2 1) proline 3 (2 9) alanine and 2 (1 9) valine residues (experimental values in brackets) Based on the amino acid composition the minimum molecular weight is 3922

Strøier Rasmussen P K Murray and J Murray Luck Biochemistry 1962 *1* 79

* Part of this work was carried out at the Department of Chemistry Stanford University California U.S.A and supported by a grant from the National Institute of Health United States Public Health Service (Grant C—484)

Svensmark, O., Human serum cholinesterase as a sialo-protein Acta physiol scand 1961 *52* 267–275
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Abstr XI Scand Physiol Congr Copenhagen 1963
Acta physiol scand 1963 59 Suppl no 213

Svensmark, O (Institute of Neurophysiology, University of Copenhagen, Denmark) PRECURSORS OF SERUM CHOLINESTERASE IN HUMAN LIVER

It is generally assumed that serum cholinesterase (acylcholine acyl hydrolase, EC 3 1 1 8) is produced in the liver as liver damage and certain liver diseases cause a decrease in serum cholinesterase activity To investigate whether liver contains the same type of enzyme as serum liver cholinesterases were studied with respect to enzymatic properties, electrophoretic mobility (*Svensmark* and *Kristensen* 1963), and effect of neuraminidase on the enzymes (*Svensmark* 1961)

Two main fractions of cholinesterase (I and II) were separated by diethylaminoethyl-cellulose chromatography of aqueous extracts of human liver tissue from which the blood was thoroughly removed One of these fractions (I) was subdivided into two fractions by gel filtration on Sephadex G-200 One fraction (Id) appeared immediately after the void volume, as did human serum cholinesterase, another fraction (Ie) was retarded on the column which indicates a molecular weight for the protein lower than that of serum cholinesterase All three fractions were identical with serum cholinesterase with respect to substrate and inhibitor specificity pH dependence and activation by ammonium ions

The electrophoretic mobilities at pH 4–9 of fraction II and serum cholinesterase were identical After enzymatic removal of sialic acid the mobility of fraction II was increased as was that of serum cholinesterase This indicates that the enzyme is a sialo-protein The electrophoretic mobilities of fraction Id and Ie were unaffected by treatment with neuraminidase showing that these enzymes are not sialo-proteins The mobilities of both fractions differed from those of native and sialic acid free serum cholinesterase

It is unlikely that fraction I originates from a degradation of fraction II since the relative activity of the fraction did not increase after standing of the homogenate at 25° C for 10 days The findings are therefore consistent with the assumption that serum cholinesterase is produced in the liver with fraction Id and Ie as precursors, and fraction II as the final product

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Thorsen, T., K F Støa and D Aarskog (Hormone Laboratory University of Bergen Norway) URINARY ALPHA KETOLIC OESTROGENS IN THE NEWBORN

Oestrogenic substances occur in the urine of newborns as shown by biological and chemical methods The main oestrogenic steroid excreted during the first 4–5 days of life is oestriol whereas oestrone and 17β-oestradiol are present in hardly detectable amounts even following intramuscular injection of relatively high doses of 17β-oestradiol (*Diczfalusy et al* 1957 *Barr et al* 1961) The role of the foetal oestrogen metabolism in pregnancy has been studied by the same group of investigators (*Diczfalusy et al* 1961) and the influence of foetal liver and brain damage or of the foeto-placental circulation on the maternal oestrogen excretion is well recognized (*Cassmer* 1959 *Frandsen* and *Stakemann* 1960 *Coyle* 1962) This indicates that the intermediary metabolism of 17β-oestradiol in the foetus and newborn is qualitatively different from that observed in the adult and that the foetal contribution to the oestrogen biosynthesis and metabolism is of importance in normal pregnancy

In most of these studies the method of *Brown* (1955) was used Consequently the interest has been mainly centered on oestrone 17β-oestradiol and oestriol However a number of other phenolic steroids enter into the metabolic sequence of the oestrogenic hormones The most important of these metabolites are 16-epioestriol and the ring D α ketolic oestrogens (*Hobkirk* 1963) By means of a spectrofluorimetric technique (*Støa* and *Thorsen* 1962) combined with column and paper chromatography determination of the total α ketolic fraction in separate 24 h urine samples as well as identification of 16α hydroxy-oestrone and 16-oxo-17β-oestradiol in pooled urine from newborn infants have been carried out. The results obtained confirm previous investigations on the oestrogen metabolism of the foetus and the newborn.

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Abstr XI Scand Physiol Congr Copenhagen 1963
Acta physiol scand 1963 *59* Suppl no *213*

Thorn, N A and N B S Willumsen (Institute of Medical Physiology, University of Copenhagen, Denmark) DIRECT AND INDIRECT EFFECTS OF HIGH CONCENTRATIONS OF UREA ON THE PERMEABILITY OF RAT KIDNEY CELLS TO WATER

During dehydration the concentration of urea in the inner zones of kidney papillae may reach values approaching those of the urine The maximum urinary concentrations reported were 1 7 M for rats and 2 5 M for carnivora We have investigated the effect of high concentrations of urea on the inactivation of antidiuretic hormone (ADH) by renal papillary tissue and on the water content of such tissue

The white papillary zones were isolated from kidney slices (0 4 mm thick) and transferred to Warburg vessels containing *Robinson*'s medium (1956) to which various concentrations of urea were added Parallel sets of slices kept in media without urea served as controls Urea in concentrations above 0 5 M consistently increased the water content of papillary slices incubated without hormone In other experiments 0 2 I U of a commercial preparation of arginine vasopressin was added to each vessel and incubated aerobically with slices for 2 h The amount of hormone inactivated was determined by bioassay after the incubation Increasing concentrations of urea caused increasing inhibition of the inactivation with 0 5 M and 1 M urea the inhibition being 10 and 50 per cent With these concentrations and short time exposure the inhibition was reversible These findings indicate an inhibition of the ADH inactivating enzyme system A corresponding effect of urea (in concentrations lower than those causing denaturation) on other enzyme systems has been reported (*Rajagopalan Fridovich* and *Handler* 1961)

It is generally assumed that urea merely plays a passive role in the concentrating mechanism by its contribution to the osmolarity of the urine depending on the permeability characteristics of the different parts of the nephron In addition to this effect urea in high concentration may interfere with the permeability to water in the most distal part of the nephron, directly and by changing the metabolism of the antidiuretic hormone

Rajagopalan K V I Fridovich and P Handler J biol Chem 1961 *236* 1059

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Acta physiol scand 1963 *59* Suppl no *213*

Tygstrup N, K Winkler and F Lundquist (Department of Biochemistry and Cardiologic Laboratory of the Medical Department B Rigshospitalet University of Copenhagen Denmark)
THE INFLUENCE OF ETHANOL ON THE HEPATIC FRUCTOSE METABOLISM IN MAN

The hepatic fructose metabolism was examined in six subjects by hepatic venous catheterization After a control period infusion of 10 mmoles fructose per min was started The splanchnic uptake of fructose averaged 2 30 mmoles/min This uptake could be partly accounted for by the following changes An increase in the output of lactate corresponding to 1 03 mmoles/min of fructose, of pyruvate corresponding to 0 16 mmoles/min and of glucose corresponding to 0 15 mmoles/min There was an uptake of sorbitol of 0 20 mmoles/min and the arterio-venous difference of glycerol was not significant Thus 1 22 mmoles of fructose per min must have been either metabolized to carbon dioxide and water or stored in the liver probably as glycogen

Fructose was added to an infusion of ethanol in 8 subjects On the average 3 01 mmoles were taken up in the splanchnic organs per min. The increase in lactate output corresponded to 0 34 mmoles the output of pyruvate decreased corresponding to 0 02 mmoles and the output of glucose rose by 1 40 mmoles per min 0 69 mmoles were given off as sorbitol and 0 10 mmoles as glycerol Thus 0 48 mmoles must have been oxidized completely or stored

As judged from the increase in the splanchnic production of carbon dioxide only a minor part is completely metabolized unless the carbon dioxide produced arises from nothing but fructose In the latter case about half of the fructose uptake not accounted for by metabolites is oxidized This applies to both types of experiment.

Comparison between the two types of experiment shows the following effects of ethanol on fructose metabolism

1) The sum of reduced metabolites (sorbitol + lactate + glycerol) and the ratio lactate/pyruvate are increased

2) The output of lactate pyruvate and carbon dioxide is reduced

3) The output of glucose is increased

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Acta physiol scand 1963 59 Suppl no 213

Ussing H H (Institute of Biological Chemistry University of Copenhagen Denmark) EFFECTS OF HYPERTONICITY PRODUCED BY UREA ON ACTIVE TRANSPORT AND PASSIVE DIFFUSION THROUGH THE ISOLATED FROG SKIN

Recent studies of the electrical potential profile across the frog skin epithelium using *Ling Gerard* micro-electrodes (*Ussing* and *Windhager* 1963) are essentially in agreement with the model proposed by *Ussing* and *Koefoed Johnsen* (1956) and *Hoshiko* and *Engbæk* (1956) but the model has to be modified in certain respects

Thus the sodium permeable and potassium impermeable membrane must be located immediately underneath the very thin cornified layer The nature of the potential profile can best be explained if it is assumed that all the living cells of the epithelium are connected through the intercellular bridges in a syncytium like fashion allowing active sodium transport inward by the cellular path whereas the interspaces allow a certain shunting of the sodium battery by diffusion of anions inward and sodium outward

That such an arrangement exists receives support from observations on the responses of the skin when the outside medium is made hypertonic by addition of urea If a skin is suspended with sulphate Ringer on both sides and urea is then added to the outside compartment to make the solution twice isotonic the d c resistance of the skin may drop to one fifth in less than one hour Tracer experiments show that the drop in resistance is due to an enormous increase in the passive diffusion of sulphate as well as sodium At the same time the short-circuit current (which is a measure of the active sodium transport) usually remains practically constant. That the shunt conductance of the skin and the shunt by passive leakage of sodium in particular can be increased so dramatically without influencing the active sodium transport markedly makes it unlikely that the shunt is established through the actively transporting cells Urea may be assumed to reduce reversibly the resistance of a barrier which normally impedes the access to the interspaces between the epithelial cells The same barrier may cover the whole outer face of the epithelium since urea elicits an increase in the water permeability of the outer barrier of the epithelium cells as evidenced

4) The calculated storage as glycogen is reduced

5) The splanchnic oxygen consumption is increased more than can be accounted for by the metabolites examined

The first two and in part the third observation may be explained on the basis of an increased NADH/NAD ratio)* in the liver Formation of glycerol phosphate probably plays an important role

* Reduced nicotineamide adenine dinucleotide/nicotineamide adenine dinucleotide ratio

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Valleala, P., H Lang and T Tuovinen (Department of Physiology University of Turku Finland) AMYGDALOID AFTER DIS CHARGE AND GALVANIC SKIN RESPONSE

This investigation was carried out on lightly anesthetized cats immobilized by succinyl choline Stimulation of amygdala and re cording of the amygdaloid after discharge (AA) were made in the basolateral part of this nucleus Galvanic skin response (GSR) was recorded from the volar surface of the fore paw by a DC amplifier The term phasic GSR is used for a reaction of short duration and sinusoidal form tonic GSR indicates a response of long duration and steady flat form In animals under light anesthesia there is also spontaneous undulation of the skin potential which in this study is called background galvanic skin activity (GSA)

When the amygdaloid stimulation was subliminal for AA GSR was phasic or absent In amygdaloid stimulations in which AA occurred GSR was tonic

The final phases of AA and tonic GSR showed a certain tem poral relation Attempts were made to abolish this relationship by pharmacological means Xylocaine® (3 mg/kg i v) was without influence in that it shortened the amygdaloid after-discharge and GSR equally The latency amplitude and tonic character of GSR remained unchanged The shortening of tonic GSR was in this case the immediate outcome of the reduced duration of the amyg-daloid discharge Nembutal® disrupted the temporal relationship between the final phases of AA and GSR in that it shortened the duration of AA less than Xylocaine® but had a pronounced effect on GSR Its duration was shortened amplitude decreased and latency lengthened The results after Nembutal® represent the only cases in which tonic GSR was absent although there was an amyg daloid after-discharge The possibility that the tonic GSR is a sec ondary phenomenon caused by projections of AA to the brain stem is discussed

by their osmotic behaviour measured by the technique of *Mac Robbie* and *Ussing* (1961)

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Acta physiol scand 1963 59 Suppl no *213*

Weis Fogh, Torkel (Zoophysiological Laboratory B University of Copenhagen Denmark) DIFFUSION IN INSECT WING MUSCLE

The fibres of insect wing muscle are striated and they often approach giant dimensions Nevertheless wing muscle represents the most active aerobic tissue known the metabolic rate correspond ing to 0 5–2 5 kWatt/kg muscle Insects lack a closed vascular system but have a highly developed system of air tubes which branch and taper profusely so that the terminal tracheoles have a diameter of only 0 1 μ The problem is to account for the enormous exchanges of respiratory gases and of fuel by means of four different me chanisms obviously at the disposal of a flying insect (1) ventilation of the tracheal system by means of respiratory movements (2) local pumping of air and blood due to the length changes of the muscles themselves (3) diffusion of gas in the air tubes and (4) diffusion of dissolved gas and fuel in blood and tissue

The large wing muscles of dragonflies (*Aeshna spp*) were found to be organized in a sufficiently regular way to make possible de tailed investigation on muscular pumping and diffusion The results were extended by comparisons with other insects

In all insects muscular pumping is essential not for moving air but for the exchange of blood (haemolymph) between the interior and the exterior of whole muscle Nevertheless the tidal character of the pumping is shown to make necessary a very high concentra tion of fuel 5–10 mg/ml in most insects The high content of trehalose or protein bound lipids or of both in winged insects is therefore probably an adaptation to flapping flight In addition some fibres are so large and active that the difference in concentra tion of carbohydrate between the plasma membrane and the centre must also reach 5–10 mg ml but no direct estimates are available

As to exchange in the tracheal system small insects like *Drosophila* depend exclusively on diffusion but in larger insects the pri mary air tubes are and must be strongly ventilated In the remaining 1–2 mm of the pathways diffusion is the only effective mechanism and is found to be sufficient because of the large hole fraction Be tween 10^{-1} and 10^{-3} of any cut surface area consists of air passages

The exchange between air tubes and the sites of consumption

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Wahlstrom, Goran (Institute of Pharmacology, University of Uppsala, Sweden) RESETTING OF THE BIOLOGICAL CLOCK CONTROLLING THE SELF-SELECTED CIRCADIAN RHYTHM IN THE CANARY

A method to study self-selected circadian rhythm in the canary has been developed A cage is used thoroughly protected from outer light and in which there are two perches When the bird sits on one of the perches (night perch) the light in the cage is extinguished The other perch (activity perch) is used to measure the activity during the light period With this method most of the birds put singly into the cages develop a circadian rhythm with only one period of activity and one of rest The circadian period consisting of activity and rest is about 24 h but often longer or shorter The waking up times are taken as start and end points of the circadian period, they are more constant than the retiring times

Experiments have been performed where the self selection situation has been temporarily abolished by extinguishing the light When these extrogenous dark periods occur within the normal activity period and thus form an interruption of it there is no change in waking up or retiring time as compared with the pre-experimental period

When a dark period is placed so as to cover one waking up time there is a definite influence on the following one If the duration of the light interruption D (calculated from the time the bird jumps off the night perch to the time the light is turned on) is shorter than 3 h, the next waking-up time is delayed by approximately D hours If D is longer than 3 h the delay is only about 3 h irrespectively of the length of D Once a delay has occurred it is not compensated later The rhythm goes on from a new starting point the clock has been reset Even with a rather long D there is no or only a slight delay of the following retiring time The delays seen in the waking up times thus could not be secondary to a primary effect on the retiring time

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The exchange between air tubes and the sites of consumption

takes place *via* the tracheoles, which must approach the mitochondria to within 5–10 μ In most cases they indent the fibre surface and form an internal network two or three times more dense than necessary *Odonata* and *Blattidae* have no internal tracheoles and the respiring cortex of the fibres is reduced to the limiting thickness of 8–10 μ The peculiar alternate, lamellar arrangement of fibrils and giant mitochondria in these insects may improve diffusion of oxygen as compared with the diffusion in an isotropic system

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Werdinius B, N E Anden and B E Roos (Department of Pharmacology University of Goteborg Sweden) ON THE OCCURRENCE OF PHENOLIC ACIDS IN BRAIN AND CEREBROSPINAL FLUID

The metabolism of the catecholamines in the brain is only partly known Dopamine is located mainly in the corpus striatum and has probably a function of its own in the extrapyramidal system Through the enzymes monoamine oxidase (MAO) and catechol O methyltransferase (COMT) both present in the brain dopamine is degradated to homovanillic acid (HVA) By the action of MAO alone dopamine is converted into 3 4-dihydroxyphenylacetic acid (DOPAC) which may in turn be converted to HVA by COMT Dopamine may also be attacked first by COMT then by MAO to form HVA

A fluorometric method for the determination of HVA in brain and cerebrospinal fluid has been developed and we have studied the effect of reserpine and some other drugs on the HVA level of the rabbit corpus striatum Various parts of the human brain were examined in necropsy material both from persons without cerebral diseases and from patients with parkinsonism

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Wiesendanger, M (Institute of Neurophysiology, University of Copenhagen, Denmark) RIGIDITY IN CHRONICALLY DEAFFERENTED CATS

Reports of the effect of chronic deafferentation of a limb upon posture and locomotion are controversial *Sherrington* (1897/98) found abolished postural tone after acute and chronic deafferenta tion in cats and monkeys *Ranson* (1928) elicited extensor hyper tonus by chronic intradural deafferentation in cats I have rein vestigated the effect of chronic unilateral deafferentation (L3–5 to S2–3) in 13 cats In seven, the dorsal roots were cut extradurally and in six intradurally Only animals with loss of sensibility tested by squeezing and pinprick were included Both intradurally and extradurally operated cats had a scoliosis convex to the side of the operation, which often interfered with standing and stepping During sitting, standing, stepping and jumping the deafferented limb was held in an extended position it often slipped backwards or side wards and touched the ground with the dorsum of the pad The tendency to keep the deafferented hind leg extended—seen both in extradurally and intradurally operated cats—increased for 3–4 weeks The scratch reflex was more readily elicited on the deaf ferented side, tonic neck- and labyrinthine reflexes were not exag gerated Only intradurally operated cats had disturbances in the function of the bladder In a standardized position (sling) in in tradurally deafferented legs, the electrical activity of the extensor muscles exceeded that of the normal side in extradurally deaf ferented legs—as in normal legs—activity in the extensor muscles was absent or slight A typical supporting reaction was absent in both but intradurally deafferented legs showed varying degrees of resistance when supported Electrical stimulation (4 pulses 0 1 msec 500/sec) of the plantar nerve on the non-deafferented side provoked a burst of activity in the flexors as well as in the extensors of the deafferented leg In the extensors of extradurally operated legs a burst of about 50 msec occurred only with high strength of stimula tion (4–8 times the threshold of the response in the flexor muscles) in intradurally deafferented legs the response evoked in the extensor muscles was more pronounced often lasted for several seconds and had the same low threshold as the response of the flexors Thus a

true hypertonus of extensor muscles is only present in intradurally deafferented cats probably due to damage to the spinal cord The occurrence of bladder disturbances is consistent with this interpretation (*Sprong* 1929)

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Abstr XI Scand Physiol Congr Copenhagen *1963*
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Wiesendanger, Mario and Arne Mosfeldt Laursen (Institute of Neurophysiology, University of Copenhagen Denmark) THE EFFECT OF THE PYRAMIDAL TRACT ON FLEXORS AND EXTENSORS IN CATS

Reports of the effect of section of the medullary pyramids are controversial A number of authors, beginning with *Magendie* (1834) found no effect on movements Other observers reported extensor hypertonus (pyramidal rigidity *Ranson* 1932), some found it most pronounced in the contralateral upper (*Ranson* 1932), others in the lower extremities (*Liddell* and *Phillips* 1946) *Marshall* (1934) and *Tower* (1936) believed the extension to be due to hypofunction of flexor reflexes

To decide whether extensor hypertonus is produced or flexor reflexes are reduced we have recorded muscle action potentials from *one to three weeks after unilateral or bilateral pyramidotomy* With the cat supported in a sling and the paws free of the ground activity was absent in both flexors and extensors as in normal cats Passive flexion elicited the same degree of activity in ipsilateral and con tralateral extensors The flexion elicited by pinching the skin of the foot appeared later and was less pronounced in the contralateral legs

That the pyramidal tract acts predominantly on flexors was confirmed by stimulation of the medullary pyramids before and after section of the rest of the medulla oblongata (*Lloyd* 1941) With the pyramids preserved the movements evoked in decerebrate *cats were complex and variable (Landau 1952) After* isolation of the pyramids one and five sec trains of rectangular stimuli (100/sec 0 1 msec) evoked short bursts of activity confined to the flexors of the contralateral forelimb

This is in agreement with the finding that the pyramidal tract in cats is distributed mainly to the cervical part of the spinal cord (*Wall McCulloch Lettvin* and *Pitts* 1956) Sensitive measures such as summation of pyramidal and monosynaptic afferent stimuli (*Lloyd* 1941), may be necessary to detect the pyramidal effect on the lumbar cord *That flexors were activated is in agreement* with the results of intracellular recording from motoneurons (*Lundberg* and *Voorhoeve* 1962)

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Willumsen, N B S and N A Thorn (*Institute of Medical Physiology, University of Copenhagen, Denmark*) INHIBITORY ACTION OF HIGH CONCENTRATIONS OF UREA ON THE OXYGEN COMSUMPTION BY RAT KIDNEY SLICES FROM CORTEX AND MEDULLA

During dehydration there is a marked concentration gradient for urea through the medulla of the rat kidney, with ensuing high concentrations of urea in the papilla The oxygen consumption of the kidney cortex (*Robinson* 1962) and of the papillary zone from rat kidney slices (*Thorn* and *Willumsen* 1963) decreased with high concentrations of urea, complete suppression being obtained in papillary tissue with 2 M urea We have compared the sensitivity of cortical and papillary slices to urea and examined the reversibility of the effect Furthermore, the inhibitory effect of urea on the oxygen consumption was compared with its effect on glycolysis

Kidney slices of 0 3–0 4 mm thickness were cut longitudinally on the Stadie-Riggs microtome and divided into zones comprising cortex, outer medulla and papilla, respectively The slices were transferred to Warburg vessels with different media containing urea in different concentrations, up to 4 M Parallel sets of slices in media without urea served as controls After equilibration and incubation with urea for 1 h the oxygen consumption per mg of dry tissue (corrected for the content of urea) was determined manometrically The oxygen consumption of slices from cortex, outer and inner medulla was inhibited at urea concentrations above 1 M There was no difference in sensitivity between cortex and medulla and the inhibition was largely irreversible

Similar concentrations of urea inhibited the enzyme system which inactivates the antidiuretic hormone (*Thorn* and *Willumsen* 1963) These findings stress the special conditions which may exist for the metabolic functions of the kidney papilla during dehydration

Robinson J R J Physiol (Lond) 1962 *164* 552
Thorn N A and N B S Willumsen 1963 This volume p 00

Abstr XI Scand Physiol Congr Copenhagen 1963
Acta physiol scand 1963 59 Suppl no 213

Winkler K, N Tygstrup and F Lundquist (Department of Biochemistry and Cardiologic Laboratory of the Medical Department B Rigshospitalet University of Copenhagen Denmark)

THE INFLUENCE OF FRUCTOSE ON THE HEPATIC CIRCULATION AND METABOLISM IN MAN

Ethanol in non toxic doses causes a slight reduction in the hepatic blood flow without affecting the splanchnic oxygen uptake In the present study we have investigated the effect of intravenous infusions of fructose (10 mmoles/min) both alone and in combination with ethanol (2 5 mmoles/min) With fructose alone (6 subjects) hepatic blood flow is insignificantly increased with fructose and ethanol together (9 subjects) there was a significant increase (30 per cent) This was found with both bromsulfalein (4 subjects) and indocyanine green (5 subjects) as indicators In two cases (not included in the material) accurate measurement of the hepatic blood flow was not possible because the clearance of bromsulfalein was reduced during fructose-ethanol infusions

Fructose alone resulted in an insignificant average increase of the splanchnic oxygen uptake (30 per cent) with fructose and ethanol in combination the increase was significant (60 per cent) The splanchnic production of carbon dioxide did not vary systematically when fructose was infused but was significantly lower during infusion of ethanol The same applies to the respiratory quotient of the splanchnic area

The total oxygen uptake of the subjects was increased by 25 per cent by fructose alone and by 38 per cent when fructose was applied together with ethanol The respiratory quotient of the subjects was not changed by infusion of ethanol or of ethanol and fructose combined With fructose alone the respiratory quotient was close to unity

Zade Oppen, A M M and T C Laurent (Institute of Physiology and Institute of Medical Chemistry, University of Uppsala Sweden) ON A POSSIBLE MODE OF ACTION OF POLYMERS ON HYPOTONIC HEMOLYSIS

Dextran and other high molecular substances inhibit hemo globin liberation in hypotonic media (*Marsden* 1954, *Marsden Zade Oppen* and *Johansson* 1957) and decreased the solubility of pro teins (*Laurent* 1963) It was investigated whether these phenomena could be related

Washed erythrocytes were lysed by freezing, and the pigment was converted to cyanmethemoglobin The hemolysate was dialyzed against 0 1 M phosphate buffer (pH 7 6) and mixed with dextran (M_w 80,000 M_n 41,000) of concentrations of 33–164 g/l The mixtures were allowed to stand at +4° C for two days and then centrifuged at 1500×G for five min The amounts of microscopically visible cell fragments in the supernatants decreased with increasing concentration of dextran After centrifugation at 60 000×G for 30 min the precipitates were recovered from each tube and analysed for nitrogen and iron About half of the nitrogen content was non hemoglobin, and the absolute amount of this fraction increased with increasing dextran concentration

The experiment suggests that non hemoglobin proteins from erythrocytes are precipitated by dextran which might interfere with the normal pattern of hemolysis

Marsden N V B Acta physiol scand 1954 *31* Suppl 114
Marsden N V B M Zade Oppen and L P Johansson Exp Cell Res 1957 *13* 178
Laurent T C Biochem J 1963 In press

Abstr XI Scand Physiol Congr Copenhagen 1963
Acta physiol scand 1963 59 Suppl no *213*

Zimmermann Nielsen, C. (Biochemical Institute University of Aarhus Denmark) IN VIVO STUDIES ON PROTEIN DIGESTION

There is uncertainty as to how long time is required for the proteolytic enzymes in the digestive tract to split the proteins into free amino acids The rate of disappearance of pure animal and vegetable proteins from the small intestines was determined in cats in acute experiments under the following conditions introduction of proteins into the small intestines with intact (1) or blocked (2) pancreatic secretion or introduction of proteins predigested with pepsin *in vitro* into the small intestines with intact (3) or blocked (4) pancreatic secretion The proteins were introduced in isonitrogenous quantities and in iso-osmotic solutions or suspensions After a suitable time the disappearance of tannic acid precipitable N and of total N from the small intestines was determined The experiments indicate that pepsin predigestion is important for the rate of disappearance of the vegetable proteins whereas in the case of animal proteins it is of minor importance The proteolytic enzymes of the pancreas accelerated the disappearance of the predigested vegetable proteins and did not influence the disappearance of the native proteins Pancreatic and intestinal enzymes influenced greatly the rate of disappearance of animal protein nitrogen from the small intestines Elimination of pancreatic secretion did not abolish digestion of animal proteins but decreased the rate of disappearance

Abstr XI Scand Physiol Congr Copenhagen 1963
Acta physiol scand 1963 *59* Suppl no *213*

Anggård, Erik and Bengt Samuelsson (Department of Pharmacology and Department of Chemistry, Karolinska Institutet Stockholm Sweden) SMOOTH MUSCLE STIMULATING LIPIDS IN SHEEP LUNG

Ethanol extracts of normal sheep lung were analyzed for smooth muscle stimulating factors The lipids were separated into acidic and neutral fractions Practically all spasmogenic activity was confined to the acidic fraction This was subjected to further purification by means of silicic acid, reversed phase partition, and thin layer chromatography Prostaglandin F α was the main smooth muscle stimulating acidic lipid This compound has previously been isolated from sheep and swine lung (*Bergstrom et al* 1962) Injected i v in doses of 15–30 ug/kg it has pronounced effects on pulmonary circulation in cats and stimulates various isolated smooth muscle organs in concentrations from 10^{9} g/ml (*Ånggard* and *Bergstrom* 1963)

Utilizing an isotope dilution method with tritium labelled prostaglandin F α the amount of this compound in sheep lung was determined to about 0 5 μg/g lung (wet weight) It was also possible to isolate prostaglandin E from sheep lung This factor was identified by its chromatographic behaviour in various systems as compared with pure prostaglandins Ultraviolet spectrophotometry and differential pharmacological assay afforded further proof as to the identity Spasmogenic activity could also be found in fractions both more and less polar than the prostaglandins These were not further characterized Details of the isolation technique will be given and the psysiological implications of the findings discussed

Änggård E and S Bergstrom Biological effects of an unsaturated trihydroxy acid (PGF α) from swine lung Acta physiol scand 1963 *58* 1–12

Bergstrom S F Dressler L Krabisch R Ryhage and J Sjovall The isolation and structure of a smooth muscle stimulating factor in normal sheep and pig lungs Arkiv Kemi 1962 *20* 63–66

Abstr XI Scand Physiol Congr Copenhagen 1963
Acta physiol scand 1963 59 Suppl no 213

Öberg Bengt (Department of Physiology University of Goteborg Sweden) ASPECTS OF THE REFLEX CONTROL OF NET CAPILLARY FILTRATION IN SKELETAL MUSCLE AND INTESTINE

A study has been performed on anesthetized cats to evaluate the reflex effects elicited from carotid sinus and cardiac receptors on net transcapillary filtration in skeletal muscle and intestine In addition reflexly induced shifts in capillary fluid exchange during hemorrhage were investigated

The reactions of the resistance vessels were studied by blood flow pressure measurements and the filtration process by continuous recording of changes in tissue volume with a plethysmographic tech nique

A lowering of carotid sinus pressure caused a marked vasoconstriction in skeletal muscle accompanied by an absorption of fluid from the extravascular space The amount of fluid absorbed was related to the degree of vasoconstriction An increase of carotid sinus pressure produced vasodilatation and outward filtra tion in skeletal muscle These experimental procedures caused simi lar changes in flow resistance in the intestine although less pronounced than in muscle and did not affect the transcapillary fluid exchange Activation of cardiac receptors with protoveratrin evoked a peripheral vascular response pattern resembling that induced by stimulation of arterial baroreceptors

Acute hemorrhage elicited a sustained reflex vasoconstriction in skeletal muscle and an absorption of extravascular fluid quanti tatively related to the magnitude of the hemorrhage In the intestine bleeding produced a slight vasoconstriction but no transfer of fluid from the extravascular compartment.

Under the prevailing experimental conditions shifts in net capil lary filtration reflect variations in capillary hydrostatic pressure in turn due to adjustments of the pre /postcapillary resistance ratio The present experiments demonstrate that there is a powerful reflex control of this ratio in skeletal muscle On the other hand such reflex adjustment is of little importance in the intestine probably due to competitive action of local autoregulatory mechanisms

In an attempt to evaluate the relative importance of carotid

sinus and cardiac receptors for inducing the peripheral vascular adjustments in hemorrhage, standardized bleedings were performed before and after blocking the afferent fibres from the respective re ceptor sites These experiments suggest that the carotid sinus re ceptors exert a relatively more powerful influence than the cardiac receptors upon the peripheral vascular beds The cardiac receptors are more important for the regulation of the heart

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VOL. 59 SUPPLEMENTUM 215

ANALYSIS OF THE Mg^{++}-ATP DEPENDENT STORAGE MECHANISM IN THE AMINE GRANULES OF THE ADRENAL MEDULLA

BY

ARVID CARLSSON, NILS ÅKE HILLARP
AND BERTIL WALDECK

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FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF GÖTEBORG
AND THE DEPARTMENT OF HISTOLOGY, KAROLINSKA INSTITUTET
STOCKHOLM, SWEDEN

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CONTI NTS

INTRODUCTION

In an earlier paper we reported that the amine granules of the adrenal me
lulla are able to take up and concentrate monoamines *in vitro* by a Mg^{++}–ATP[1]
lependent storage mechanism which is blocked by low concentrations of
eserpine (CARLSSON, HILLARP and WALDECK 1962). Independent work by
KIRSHNER (1962 a and b) yielded similar results. The significance of these
observations is emphasized by the fact that the transmitter of the adrenergic
nerves is stored in granules with similar properties (EULER and HILLARP 1956,
EULER 1958, EULER and LISHAJKO 1961, SCHUMANN 1958). In fact, EULER
and LISHAJKO (1963) recently discovered a Mg^{++}–ATP dependent uptake of
NA by these granules. This uptake was blocked by low concentrations of
reserpine.

In the present paper the uptake mechanism is analyzed in further detail.

[1] Abbreviation: DA = 3,4-dihydroxyphenylethylamine (dopamine), NA = noradrena
line, A = adrenaline, 5-HT = 5-hydroxytryptamine, ATP, ADP, AMP = adenosine tri-,
... monophosphate respectively.

MATERIAL AND METHODS

Isolation of the medullary granules Amine granules from 3 to 5 cow adrenal medullas were isolated in 0.3 M sucrose (HILLARP 1958) The granules were not washed but the looser layer above the more tightly packed bottom sediment (after centrifugation at 26 000 × *g* for 20 min) was removed by swirling with sucrose Finally the granules were suspended in 0.3 M sucrose and stored at 0° They were used the same and the next day

Standard conditions for C^{14} amine uptake An aliquot (50 μl) of the granule suspension, corresponding to 70 to 90 μg of granule bound and 15 to 20 μg of free catecholamines (wet wt of granules about 2 mg) was transferred to 1.0 ml of an incubation mixture (at 0°) containing 0.31 M glycylglycine (pH 7.3 with NaOH) 0.0025 M ATP and $MgCl_2$ 25 μg unlabelled L-NA (or L-A) and 2.5 to 5 μg C^{14}-NA (or C^{14}-A) Sometimes a 0.17 M histidine HCl buffer (pH 7 to 7.3) was used Incubation was performed without shaking at +31° and 0° (controls) for 30 min The tests were practically always made in duplicates

After the incubation the suspension was chilled to 0° diluted about 30 times with cold 0.3 M sucrose and — after about one hour at 0° — centrifuged at 100 000 × *g* for 30 min After thorough rinsing of the tubes with 0.3 M sucrose the granule sediment was extracted with 5.0 ml of 0.01 N HCl in 98% ethyl alcohol

Preincubations The effect of several compounds was tested by a preincubation of the granules with the compound 0.5 ml of the granule suspension was added to 8 ml 0.3 M sucrose containing the agent to be tested (concentration see the tables) Incubation was performed for 30 min at 0° or +31° The suspension was then centrifuged at 26 000 × *g* for 15 min at 0° The granules were resuspended in 0.4 ml 0.3 M sucrose Their capacity to take up C^{14} amines was tested by adding aliquots (50 μl) to the standard incubation medium described above The uptake was in every case compared with that of control granules treated in the same way

Determinations of catecholamines and C^{14} compounds The catecholamine content of the extracts was determined spectrophotofluorimetrically (BERTLER CARLSSON and ROSENGREN 1958) The C^{14} amine content was determined either directly in a liquid scintillation counter or after the catecholamines had

been taken up on a small cation exchanger (Dowex 50) and eluted with N HCl The eluate was evaporated to dryness *in vacuo* and the residue extracted with the acid alcohol The extract was then used for counting The identity of the C^{14} compounds was checked by paper chromatography

In the experiment with C^{14} ATP (Table 8) the granule sediments were extracted with 0 4 N perchloric acid at 0 The extracts were immediately centrifuged in the cold and — after addition of 0 5 mg sodium ethylenediamine tetraacetate — neutralized with potassium carbonate to about pH 6 at 0 After removal of the precipitated potassium perchlorate the extract was passed through a cation exchange column (Dowex 50 W 200—400 mesh 20 5 mm pH 6) The effluent and washings were brought to about pH 9 with ammonium hydroxide and passed through an anion exchange column (Dowex 1-X4 200—400 mesh 100×6 mm Cl^-) The elution of the nucleotides was performed with 0 01 (AMP) 0 06 (ADP) and 1 N HCl (ATP) and was followed by ultraviolet absorption measurements The fractions were evaporated to near dryness The residue was dissolved in a small amount of water and the extract was used for counting after dilution with alcohol — The catecholamines taken up by the cation exchanger were eluted with N HCl The eluate was treated as described above to determine the content of C^{14} amines

The purity and specific activity of the C^{14} ATP used were determined by anion exchange chromatography as described above Small amounts of C^{14} ADP and AMP were present Control chromatographies showed that the nucleotide recovery was more than 90% and that no appreciable breakdown of ATP occurred

Substances used Pure nucleotides from the Pabst Laboratories and pure reserpine generously supplied by Ciba Ltd (Basel) were used Solutions of reserpine were freshly made in acetic acid according to the method of HESSHORE and BRODIE (1956)

Monoamines with one of the sidechain carbon atoms labelled with C^{14} (specific activities ranging from 1 to 22 mC mM) were obtained from California Corporation for Biochemical Research Los Angeles (DL-adrenaline dopamine tyramine) Commissariat à l'Energie Atomique France (DL-adrenaline DL-noradrenaline) and Radiochemical Centre Amersham England (5 hydroxytryptamine) ATP 8-C^{14} (3 5 μC mg) was obtained from Schwarz Bioresearch Mount Vernon N Y

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After the incubation the suspension was chilled to 0° diluted about 30 times with cold 0 5 M sucrose and — after about one hour at 0° — centrifuged at 100 000×g for 30 min After thorough rinsing of the tubes with 0 3 M sucrose the granule sediment was extracted with 5 0 ml of 0 01 N HCl in 98% ethyl alcohol

Preincubations The effect of several compounds was tested by a preincubation of the granules with the compound 0 5 ml of the granule suspension was added to 8 ml 0 3 M sucrose containing the agent to be tested (concentration see the tables) Incubation was performed for 30 min at 0 or +31 The suspension was then centrifuged at 26 000×g for 15 min at 0 The granules were resuspended in 0 4 ml 0 3 M sucrose Their capacity to take up C^{14} amines was tested by adding aliquots (50 µl) to the standard incubation medium described above The uptake was in every case compared with that of control granules treated in the same way

Determinations of catecholamines and C^{14} compounds The catecholamine content of the extracts was determined spectrophotofluorimetrically (BERTLER CARLSSON and ROSENGREN 1958) The C^{14} amine content was determined either directly in a liquid scintillation counter or after the catecholamines had

I Activation by nucleotides and metals

A small but significant uptake occurred without addition of MgATP at +31 (Fig 3 to 5) It was inhibited almost completely by reserpine and is thus considered to be specific This uptake may well be ATP dependent since small amounts of ATP derived from the amine granules and mitochondria may be available for the specific uptake mechanism

Addition of ATP alone — but not of Mg^{++} alone — gave increased uptake but a high amine incorporation was obtained only when both had been added (Fig 4) The MgATP activation could be up to twentyfold but varied with different granules and usually was five to eight fold Mg^{++} could be replaced by Mn but not by Ca^{++} (Fig 3 to 5) In the presence of 0 00 5 M MgATP the same concentration of Ca^{++} gave no significant inhibition (Fig 3) This is of interest since it has been reported that Ca^{++} produces a release of the granule amines (HILLARP and SCHÜMANN 1962)

The specificity of the nucleotide requirement is not clear Inosine triphosphate gives the same activation as ATP but guanosine uridine and cytidine triphosphates stimulate to a lower extent (CARLSSON HILLARP and WALDECK 19) The presence of transphosphorylases cannot be ruled out however ATP could be replaced by deoxy ATP but not by sodium tripolyphosphate AMP showed no and ADP no or very small activation and did not inhibit when present in the same concentration as that of ATP (Fig 3) Adenosine 3 phosphate and phosphocreatine — with or without ATP — showed no effect (Tables 2 and) No inhibition was obtained by adenine adenosine and several other purine derivatives when present in the same concentration as ATP (Table) On the other hand 6 mercaptopurine and 8 azaguanine in the same concentrations caused about 50 per cent inhibition

The amine uptake was optimal at 0 001 M Mg^{++} and ATP when the incubation time was 15 minutes A maximal activation occurred already with 0 001 M Mg^{++} at both 0 00 and 0 00 M ATP (Fig 1) At this Mg^{++} concentration the uptake was maximally stimulated by 0 001 M ATP (Fig 2) — Under the standard conditions (31 30 min) 0 00 to 0 005 M MgATP gave optimal uptake

Since Mg may complex with 3 4 dihydroxyphenethylamines it might be possible that Mg is necessary for an association not with ATP but with the amines However the Mg^{++} concentration producing maximal activation was the same at both low and high levels of external amines (data not shown)

The dependence of the uptake mechanism on the presence of divalent metals forming ATP complexes with high association constants and the fact that the amines occur in the granules together with an equivalent amount of

RESULTS AND DISCUSSION

When not otherwise stated the amine uptake was studied under the standard conditions described above. The amount of granules (wet wt. about 2 mg) added to 1.0 ml incubation medium corresponded to 70 to 90 μg bound catecholamines (about 70% A and 30% NA) and 15 to 20 μg free amines (released from the granules at their suspension). The concentration of C^{14}-NA (or C^{14}-A) was usually 2.5 to 5 μg/ml. Unlabelled L-NA (or L-A) was usually added to the medium so that the total external concentration of catecholamines became about 50 μg/ml. The monoamine oxidase (MAO) activity of the granule suspension was so low that the use of a MAO inhibitor was unnecessary.

The storage mechanism can be blocked by *i.a.* reserpine (Kirshner 1962; Carlsson, Hillarp and Waldeck 1962) which therefore was used to test the specificity of the amine uptake in the experiments where deviations from the standard conditions were made. Other particles (*e.g.* mitochondria) present in the granule suspension take up or adsorb only insignificant amounts of catecholamines (Carlsson, Hillarp and Waldeck 1962).

Only small amounts of C^{14}-amines were found in granules incubated at 0° (Fig. 4 and 5). This uptake is probably unrelated to the specific storage mechanism since about the same amounts were recovered in sediments from granules ruptured by osmotic lysis. This view receives further support by the failure of MgATP to increase and of reserpine to block the uptake at 0° (Fig. 5). When not otherwise stated the value obtained in the 0° controls has been used as blank which has been subtracted from the values observed in the incubation at elevated temperatures. To calculate the absolute uptake the values were multiplied by the ratio of A+NA to C^{14}-amine in the medium. This may give too low values on account of a preferential uptake of the L-form of the added C^{14}-amine (see Section II). Examples of observed and calculated uptake are found in the figures.

The tests were practically always made in duplicates which showed good agreement in the values obtained (see Fig. 3, 5 and 6). All experiments of importance were repeated with different samples of granules and often with both histidine and glycylglycine buffers. The figures for per cent inhibition in the tables are mean values. In the cases where no effect of an agent was observed only the highest concentration tested is recorded in the tables.

I Activation by nucleotide and metals

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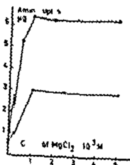


Fig 1

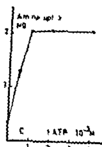


Fig 2

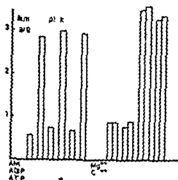


Fig 3

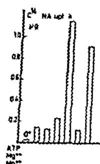


Fig 4

Fig 1 Influence of $MgCl_2$ concentration on amine uptake

Two different *samples of granules were used The same result was obtained* when the incubation time was 15 min Histidine—HCl pH 7 ATP 0 00.. or 0 00 M (upper and lower curve respectively) C^{14}—NA 15 or 6 µg/ml +31 30 min

Fig 2 Influence of ATP concentration on amine uptake

Histidine—HCl pH 7 $MgCl_2$ 0 001 M C^{14}—NA 5 6 µg/ml unlabelled NA 50 µg m +31 15 min

Fig 3 Influence of adenosine phosphates Mg^{++} and Ca^{++} on amine uptak

Two different samples of granules were used Left diagram histidine—HCl pH 7 nucleotides and $MgCl_2$ 0 00.. M C^{14}—A 7 µg/ml unlabelled A 2 µg/ml +31 30 min Right diagram glycylglycine pH 7 3 ATP $MgCl_2$ and $CaCl_2$ 0 00 5 M C^{14}—NA µg/ml unlabelled NA 25 µg/ml +31 30 min

Fig 4 Influence of ATP Mg^{++} and Mn^{++} on amine uptake

Histidine—HCl pH 7 ATP $MgCl_2$ and $MnCl_2$ 0 00 5 M C^{14}—NA 6 µg/ml no unlabelled amines added +31 30 min

TABLE 1 Fffect of enzyme inh bitors

I h bitor	Conc of I ih bitor M	Per cent Inhibi t on
Nethylmal mi l	ɔ×10⁻	4
	10 ⁴	6
All in	⁹×10⁻⁴	ɔ0
	10⁻	8
Ire ncub 0	10⁻⁴	0
Dietl yith osulfonate	1 3×10⁻⁴	⁹
	6 5×10⁻	
B Iy lroxy benzoi		
a 1 tl iosulfonat	⁹ ×10⁻⁴	40
	1 35×10⁻	⁻0
	⁹ ×10⁻⁴	85
10 M glutatl io e	10⁻⁴	ɔ
	⁹ 10⁻	9
o I 1 ben ac 1	ɔ×10 ⁴	10
	10 ⁴	1
Iodoacetam le	10 ³	0
N I a n re it	10 ³	0
N I m lf te	×10⁻⁴	0
M re m I lor 1	10⁻	⁹ɔ
	10	9
	10	9
Ire ncub 0	10 ⁴	8
p Cl m reu ibenz ate		
Pre n ct 0	10	100
Cupri 11 ei I	10 ⁴	0
Z 11 n I	10	0
Al min u hlori l	10	0
3 D merc pto 1 propa l		
BAL)	10	40
	10	80
	10 ⁴	85
Preincul 0	10 ⁴	30
Men done meth yl 1 4		
napht q one)	⁹ 10	40
	ɔ 10	0
	10 ³	9
Menad ne sod u n l s lf te	5 10 ⁴	0
Dicoumarol	1 ɔ 10	3)
	3 10	5
	3 10 ⁴	5

Inhib tor	
ɔ Hyd oxy 1 4 naphto qu none	(
1 4 Naphtoquinone	0
	1
	⁹
Preincub 0	
Preincub 0	
1 ⁹ N phtoqu none	⁹
	1
Ub qu none	μg/m
1 4 Benzoquinone	
⁹ 4 Dinitroph nol	5
Guanidine	1 5
Sodium azide	2
Sodium tripolyphosphate	⁹ 5
Sod um pyropl ospl ate	
Sodium fluoride	2
Potass um cyanide	
Anaerob incubation	
L Thyrox n	
Sodium amytal	2
	5
Cram d n D	ɔ0 μ
	100 μ
Il nol	
Pentacl loropl nol	⁹ ×
	2×
	ɔ×
I enta hloron trobenz n	⁻×
	3 5×
	⁻×
Hexachlorocyclohexan (gamma isom r)	3 ×1
Caffe ne	1

TABLE 1 *(contd.)*

Inhibitor	Conc of Inhibitor M	Per cent Inhibition
usopropy lfluorophosphate	10^{-5}	5
	10^{-4}	20
ronyl acetate	5×10^{-5}	0
micarbazide	5×10^{-3}	0
Hydroxyquinoline	5×10^{-4}	10
	10^{-3}	20
uaeilamine	2×10^{-4}	5
	10^{-3}	15
hy drine	2×10^{-5}	0
	10^{-4}	5
Acetic anhydride	2×10^{-4}	10
	10^{-3}	35
Phenylisocyanate	2×10^{-4}	0
	10^{-3}	50
Polyphloretin phosphate	25 μg/ml	15
	50 μg/ml	40
	100 μg/ml	65
Ouabaine	10^{-3}	0

ATP might suggest that such a metal is a component of the storage structure. This possibility was examined in experiments where $MgCl_2$ was replaced by $Mn^{54}Cl_2$. An ATP activated uptake of Mn^{54} was found but there was no quantitative relation between this uptake and that of the amines and it was not inhibited by reserpine. Furthermore, the mechanism responsible for the metal uptake was very labile and was destroyed soon after the preparation of the granules when amine uptake still was undiminished. Paper chromatography of the metals obtained from granules after ashing showed that the content of Mg, Mn, Ca and Zn was too low to satisfy the hypothesis.

TABLE 2 Effect of various purine derivatives

Compound	Conc of Compound M	Per cent Inhibition
...ne	2×10^{-3}	10
...osine	2.5×10^{-3}	10
...osine 5' phosphoric ...d	2×10^{-3}	0
...osine 5' diphosphoric ...d	2.5×10^{-3}	0
...osine 3' 5' phosphoric ... (with or without ...)	2.5×10^{-3}	0
...adenine	2.5×10^{-3}	20
8 Azahypoxanthine	2.5×10^{-3}	0
8 Azaguanine	2.5×10^{-3}	55–60
	6×10^{-3}	60
8 Aza 2 6 diketo 1 3 dimethylpurine	2×10^{-3}	0
6 Mercaptopurine	10^{-}[illegible]	[illegible]
	2×10^{-3}	60
2 Mercapto 6 aminopurine	2×10^{-3}	10
Trimethylxanthine	10^{-3}	0

When the amine uptake was performed in the presence of P^{32} labelled inorganic phosphate no significant incorporation of P^{32} in the adenosinephosphates of the granules occurred. KIRSHNER (1962) has reported the same result and also found no uptake of C^{14} ATP in the granules. In an experiment made in this laboratory before his data were published granules were incubated in a medium containing both C^{14} NA and C^{14} ATP (Table 8). The amines and adenosinephosphates in the granules were isolated and their C^{14} content determined. Table 8 shows that the granules contained significant amounts of C^{14} labelled ATP, ADP and AMP. However, calculated in equivalents this uptake is considerably smaller than the amine uptake. Furthermore reserpine completely blocked the amine incorporation but inhibited the uptake of C^{14} ATP only about 30 per cent.

II. Uptake of L- and D-amines

Since C^{14} NA and C^{14} A were available only in the D,L-form an indirect approach was tried to study the stereospecificity of the uptake mechanism. Granules were incubated in a medium containing a constant concentration of C^{14} NA or C^{14} A but varied concentrations of unlabelled L- or D-A. Fig. 6 shows that the dilution with the D-form decreased the uptake of the C^{14} amines considerably less than the dilution with the L-form. The result may be explained in three ways: 1) The L-form is not taken up and is an inhibitor of the uptake of the D-form, 2) the D-form is not taken up and is an inhibitor of the uptake of the L-form, 3) the mechanism is not specific but has preference for the L-form. Since the granules *in vivo* normally store the L-form the first alternative seems out of question. However, it has been shown (ANDÉN [illegible]) that both D- and L-NA are taken up by the mouse heart *in vivo* thereby displacing the NA of the granules in the adrenergic nerve terminals. This argues strongly for the third alternative.

III. Uptake of DA, NA, A, 5-HT and tyramine

No obvious differences in the uptake and in its blocking by various agents (*e.g.* reserpine, adrenergic blocking agents) were observed when C^{14} DA, NA or A were used. Uptake as function of external amine concentration was about the same when the concentration of C^{14} DA or C^{14} A was varied or when C^{14} NA was diluted with either NA or A. No careful quantitative comparisons have been made, however.

The C^{14} labelled compounds in extracts of granules incubated with C^{14} DA

TABLE 1 *(contd)*

Inhibitor	Conc of Inhibitor M	Per cent Inhibition	Inhibitor	Conc of Inhibitor M	Per cent Inhibition
Diisopropylfluorophosphate	10^{-5}	5	Acetic anhydride	2×10^{-4}	10
	10^{-4}	20		10^{-3}	35
Uranyl acetate	5×10^{-5}	0	Phenylisocyanate	2×10^{-4}	20
Semicarbazide	5×10^{-3}	0		10^{-3}	50
8 Hydroxyquinoline	5×10^{-4}	10	Polyphloretin phosphate	25 µg/ml	25
	10^{-3}	20		50 µg/ml	40
Penicillamine	2×10^{-4}	5		100 µg/ml	65
	10^{-3}	15	Ouabaine	10^{-3}	0
Ninhydrine	2×10^{-5}	0			
	10^{-4}	5			

ATP might suggest that such a metal is a component of the storage structure This possibility was examined in experiments where MgCl was replaced by $Mn^{54}Cl$ An ATP activated uptake of Mn^{54} was found but there was no quantitative relation between this uptake and that of the amines and it was not inhibited by reserpine Furthermore the mechanism responsible for the metal uptake was very labile and was destroyed soon after the preparation of the granules when amine uptake still was undiminished Paper chromatography of the metals obtained from granules after ashing showed that the content of Mg Mn Ca and Zn was too low to satisfy the hypothesis

TABLE 2 Effect of various purine derivatives

Compound	Conc of Compound M	Per cent Inhibition	Compound	Conc of Compound M	Per cent Inhibition
Adenine	2×10^{-3}	10	8 Azahypoxanthine	2×10^{-3}	0
Adenosine	2×10^{-3}	10	8 Azaguanine	2×10^{-3}	[illegible]
Adenosine 5 phosphoric acid	2.5×10^{-3}	0		6×10^{-3}	60
Adenosine diphosphoric acid	2.5×10^{-3}	0	8 Aza 2 6 diketo 1 3 dimethylpurine	2.5×10^{-3}	0
Adenosine 3 5 phosphoric acid (with or without ATP)	2×10^{-3}	0	6 Mercaptopurine	[illegible]	[illegible]
8 Azaadenine	2×10^{-3}	20		2×10^{-3}	[illegible]
			2 Mercapto 6 aminopurine	2×10^{-3}	10
			Trimethylxanthine	10^{-3}	0

When the amine uptake was performed in the presence of P^{32} labelled inorganic phosphate no significant incorporation of P^{32} in the adenosinephosphates of the granules occurred KIRSHNER (1962) has reported the same result and also found no uptake of C^{14} ATP in the granules In an experiment made in this laboratory before his data were published granules were incubated in a medium containing both C^{14} NA and C^{14} ATP (Table 8) The amines and adenosinephosphates in the granules were isolated and their C^{14} content determined Table 8 shows that the granules contained significant amounts of C^{14} labelled ATP ADP and AMP However calculated in equivalents this uptake is considerably smaller than the amine uptake Furthermore reserpine completely blocked the amine incorporation but inhibited the uptake of C^{14} ATP only about 30 per cent

II Uptake of L and D amines

Since C^{14} NA and C^{14} A were available only in the D L form an indirect approach was tried to study the stereospecificity of the uptake mechanism Granules were incubated in a medium containing a constant concentration of C^{14} NA or C^{14} A but varied concentrations of unlabelled L- or D A Fig 6 shows that the dilution with the D form decreased the uptake of the C^{14} amines considerably less than the dilution with the L-form The result may be explained in three ways 1) The L-form is not taken up and is an inhibitor of the uptake of the D form 2) the D form is not taken up and is an inhibitor of the uptake of the L-form 3) the mechanism is not specific but has preference for the L-form Since the granules *in vivo* normally store the L-form the first alternative seems out of question However it has been shown (ANDÉN 1962) that both D and L-A are taken up by the mouse heart *in vivo* thereby displacing the NA of the granules in the adrenergic nerve terminals This argues strongly for the third alternative

III Uptake of DA NA A 5 HT and tyramine

No obvious differences in the uptake and in its blocking by various agents (i e reserpine adrenergic blocking agents) were observed when C^{14} DA NA or A were used Uptake as function of external amine concentration was about the same when the concentration of C^{14} DA or C^{14} A was varied or when C^{14} NA was diluted with either NA or A No careful quantitative comparisons have been made however

The C^{14} labelled compounds in extracts of granules incubated with C^{14} DA

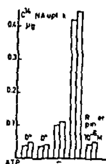


Fig 5

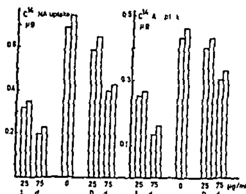


Fig 6

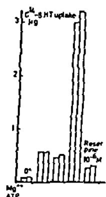


Fig 7

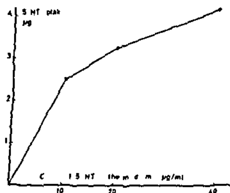


Fig 8

Fig 5 Influence of Mn^{++} on amine uptake

Histidine—HCl pH 7 ATP 0 00 5 M $MnCl_2$ 0 001 M C^{14}—N A , 6 μg/ml total external A+N A 0 0004 M +31 30 min

Fig 6 Influence of L and D adrenaline on C^{14}—amine uptake

Two different samples of granules were used Left diagram C^{14}—N A 5 6 μg/ml Right diagram C^{14}—A 4 3 μg/ml Standard conditions The concentration of external amines was varied by adding L or D adrenaline (25 and 75 μg/ml)

Fig 7 Uptake of C^{14}—5HT

Glycylglycine pH 7 3 ATP and $MgCl_2$ 0 00 5 M C^{14}—5HT 5 μg/ml no unlabelled amines were added +31 30 min

Fig 8 Uptake of 5—HT as function of external amine concentration

Glycylglycine pH 7 3 ATP and $MgCl_2$ 0 005 M C^{14}—5HT 2 5 μg/ml +37 10 min The concentration of 5—HT was varied by adding unlabelled 5—HT

TABLE 3 Effect of miscellaneous agents interfering with adrenergic mechanisms

Drug	Conc of Drug M	Per cent Inhibition
Adrenergic blocking agents		
Phenoxybenzamine	$0.33\times10^{-}$	35
	$1.6\times10^{-}$	[illegible]5
	8×10^{-5}	80
Preincub +31	$\times10^{-5}$	10
Dibenamine	$1.7\times10^{-}$	10
	8×10^{-5}	50
Preincub +31	7×10^{-5}	0
SY [illegible]8 (N-α-naphthylmethyl-N-ethyl-β-bromoethylamine)	5 µg/ml	15
	[illegible]5 µg/ml	5
	50 µg/ml	3
Phentolamine	$10^{-}-10^{-4}$	0
Preincub +31	6×10^{-5}	0
Ilidar	1.4×10^{-4}	90
	7×10^{-4}	
Ergotamine	4×10^{-6}	50
Dihydroergotamine	[illegible]$\times10^{-5}$	90
3,4-Dichloroisopropylnoradrenaline (DCI)	$1.7\times10^{-}$	35
	$3.5\times10^{-}$	50
	10^{-}	85
Preincub +31	$\times10^{-}$	0
α-Methyl DCI (H 13/6[illegible])	10^{-}	45
Phenoxyanaloge of DCI (H 13/[illegible]2)	1.7×10^{-4}	35
	$3.5\times10^{-}$	45
	10^{-}	90
L-1-(o-chlorophenyl)-isopropylaminoethanol	$\times10^{-5}$	[illegible]0
	4×10^{-5}	5
	10^{-4}	30
D-1-(o-chlorophenyl)-isopropylaminoethanol	$2\times10^{-}$	
	4×10^{-4}	30
	10	55
Miscellaneous drugs		
Nylidrin	[illegible]$\times10^{-}$	50
	10^{-}	8
Segontin	$6\times10^{-}$	4
	$1.5\times10^{-}$	5
	$6\times10^{-}$	95

Drug	Conc of Drug M	Per cent Inhibition
Imipramine	3×10^{-5}	0
	$1\ \times10^{-4}$	[illegible]5
N-monodemethylated imipramine	3×10^{-5}	[illegible]5
	1.5×10^{-4}	100
Promethazine	2×10^{-5}	40
	4×10^{-5}	65
	2×10^{-4}	9
Papaverine	10^{-5}	0
	10^{-4}	60
Quinidine	$1\ \times10^{-5}$	25
Quinine	$7\ \times10^{-5}$	[illegible]0
Lysergic acid diethylamide	$0.3\times10^{-}$	0
	3×10^{-5}	0
Cocaine	5×10^{-4}	[illegible]5
	10^{-}	40
Bretylium tosylate	2.5×10^{-4}	5
	[illegible]$\times10^{-4}$	10
TM 10 (choline 6 xylyl ether bromide)	µg/ml [illegible]0–50	0
Guanethidine	$4\times10^{-}$	10
	8×10^{-4}	15
Apresoline	3×10^{-4}–	
	1.5×10^{-4}	0
Sodium nitrite	10^{-3}	0
Monoamine oxidase inhibitors		
Tranylcypromine	2×10^{-4}	50
	5×10^{-4}	6[illegible]
	10^{-3}	95
Iproniazid	10^{-}	0
Preincub +31	10^{-3}	0
Nialamide	[illegible]5×10^{-4}	0
	[illegible]$\times10^{-4}$	5
Catron (JB 516)	$2\times10^{-}$	1[illegible]
	[illegible]$\times10^{-4}$	40
	10^{-}	60
1-Phenyl-3-hydrazinobutane (JB 835)	3×10^{-4}	15
	6×10^{-4}	25
Harmaline	10^{-5}	55

or C^{14} A were examined by paper chromatography followed by scanning the chromatogram for radioactivity. Using labelled A C^{14} was present only in the A spot. When C^{14} DA was used however, C^{14} was present mainly in the DA spot but also in the NA spot (10%). This conversion of DA to NA has also been observed by KIRSHNER (1962).

By experiments *in vitro* it has been shown that the storage mechanism is unspecific in the sense that granules normally storing only A can take up and store not only DA, NA and A but also 5 HT (BERTLER, HILLARP and ROSENGREN 1960). Exchange experiments with granules *in vitro* have also shown that DA (BERTLER *et al.* 1961), tyramine (SCHUMANN and PHILIPPU 1961) and several other phenyl and indole alkylamines including 5 HT (CARLSSON and HILLARP 1961) can replace the endogenous catecholamines at the storage sites.

The unspecificity of the storage mechanism is even better shown in experiments where granules were incubated with C^{14} 5 HT (Fig. 7 to 9). The uptake was greatly activated by MgATP and almost completely blocked by reserpine. The uptake as function of time and external amine concentration showed similar characteristics as that of the catecholamines (see Sections IV and V). The uptake mechanism seems to have a very high affinity for 5 HT. In spite of the presence of about 20 μg/ml of A+NA in the medium and only 5 μg/ml of C^{14} 5 HT the granules took up not less than about 60 per cent of the added 5 HT under the standard conditions (Fig. 7). An uptake of this magnitude has never been observed with C^{14} catecholamines (compare with Fig. 4 which illustrates an experiment with a high uptake of C^{14} NA).

Tyramine is also taken up by the granules (SCHÜMANN and PHILIPPU 1961; CARLSSON and HILLARP 1961). Experiments with C^{14} tyramine in the present work showed that the uptake differs from that of the catecholamines however. It was only slightly activated by MgATP and reserpine (up to 5×10^{-6} M) did not block the incorporation (Fig. 10). This further supports the view that tyramine is taken up — at least mainly — by displacing the catecholamines in the granules from their storage sites.

IV. Uptake as function of external amine concentration

The uptake mechanism operates efficiently at very low concentrations of external amines. Since the granule suspension contained free amines (see above) observations have not been made at levels below 20 μg/ml where up to 20 per cent could be taken up in 30 min at +31° (which is far below the temperature optimum, see Section V). The high efficiency is illustrated in an experiment with C^{14} 5 HT (Fig. 7) which is also taken up through a MgATP dependent mechanism blocked by reserpine (see Section III). In this case the

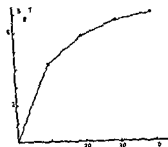


Fig 9

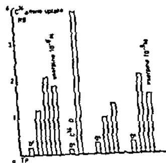
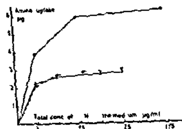


Fig 10



F 11

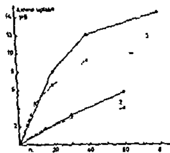


Fig 1

F g 9 Uptak of 5—HT as function of time

Glycylglycine pH 7 3 ATP and MgCl 0 002 M C — 5HT 5 µg/ml unlabelled 5—HT 40 µg ml +3

Fig 10 Uptake of C — tyramine

Three experiments with three diffe ent samples of granules Hi tidine—HCl pH ATI and MgCl 0 005 M C — tyram ne 4 µg/ml (C — DA 50 µg/ml) no unlabelled am nes added +31 30 min

F g 11 Uptake of NA as function of external amine concentratio 1

Three xperim t w th three different sampl s of granules Gly ylgly ine pH 3 ATP nd MgCl 0 00 5 M C — NA 5 µg/ml +37 30 min The concentratio 1 of external am nes was ied by dd ng unlabelled L—NA

Fig 1 Am ne uptake as function of time

Four exp rim t with four different samples of granules Lower curves standard cond t o Upper curves gly ylglycine pH ATP 0 005 M MgCl$_2$ 0 00 5 M C — A or C NA 5 µg ml unlabell d A or NA 100 µg/ml +37

C^{14} 5 HT concentration was only 5 $\mu g/ml$ (no unlabelled 5 HT) The granules took up about 60 per cent in 30 min at +31°

With increasing concentration of external amines the absolute uptake increased at first rapidly and then very slowly (Fig 11) Thus the uptake mechanism seems to be saturated at about 4 to 5×10^{-4} M The same result was obtained with DA, NA and A and under other conditions (phosphate buffer shorter incubation time higher MgATP concentration see CARLSSON HILLARP and WALDECK 1962)

In the experiments with drugs enzyme inhibitors etc the amine concentration usually was 3×10^{-4} M At this level and under the other standard conditions used the uptake seldom exceeded 10 per cent of the external amines

Reserpine 10^{-6} M *almost completely blocked the uptake at levels of external* amines up to 5×10^{-4} M (Fig 5)

At 3×10^{-4} M external amines the uptake was proportional to the granule amount between half and twice the amount used under standard conditions

V Uptake as function of time and temperature

At +31° the rate of amine uptake usually was nearly constant up to 30 min and then declined more or less rapidly Different samples of granules varied considerably in this respect as seen in Fig 12 The decline was not prevented by higher ATP concentrations It was more rapid at +37 A progressive loss in uptake ability also occurred when granule suspensions were kept at 0° This loss is marked after 24 hours and considerable after 48 hours The incorporation mechanism is thus rather labile which may be one explanation to the great variability in uptake shown by different samples of granules (see Fig 1 and 11) (cf CARLSSON HILLARP and WALDECK 1962)

In some experiments the uptake was studied as function of temperature It increased three to five times between +21 and +31 and about two times between +31 and +38° Since the magnitude of the specific uptake at 0 *is somewhat uncertain calculations of the temperature coefficient are met with* difficulties The temperature dependence must be high however (cf KIRSHNER 1962)

VI Various factors (pH buffers etc) influencing the uptake

Of the four buffers tested phosphate tris (hydroxymethyl) aminomethane HCl histidine *HCl and glycylglycine NaOH the last two gave the highest and* most prolonged uptake when used as the main osmotic constituent of the medium Mixtures of the buffers with sucrose or KCl were inferior

The pH optimum seemed to be about 7 3 when ascorbic acid was added to the medium to prevent oxidation of the amines The uptake declined rapidly below pH 7 both with and without ascorbic acid With 0 31 M glycylglycine pH 7 3 which consistently gave a higher uptake than histidine pH 7 to 7 3 no oxidation of the amines was observed which might interfere with the calculations of the uptake The uptake (+31 and +37 30 min) was the same with or without the presence of reducing agents (0 00* M ascorbic acid or 0 00*5 M sodium sulfite) No unspecific adsorption of C^{14} labelled oxidation products to the granules seemed to occur since the C^{14} content of the granule sediments was almost completely reduced to the level of the 0 controls by reserpine (10^{-6} M)

No consistent effect of Na^+ or K^+ (alone or in combination) was observed but incubation media totally free of both ions were not tested

No decrease in the uptake occurred under anaerobic conditions KCN (10^{-3} M) had no effect (Table 1)

Borate (10^{-3} M) which forms a complex with catecholamines was without effect (Table 4)

TABLE 4 Eff ct of agents interfering with cholinerg c and other mechanisms.

Co npound	Conc of Compound M	P r cent Inhibi tion
tigmi e	10 ⁴	0
eostigm ne	10 —10⁻	0— 0
p ne	10⁻ —10⁻	0—1◡
bach l n	10 ⁴—10⁻⁴	0
achol ne —		
0 3 M Ca +	×10⁻	5
pti e	10⁻ —10⁻	0
amethon um	10⁻ —10⁻	0
uboc u a ine		
lo ide	10⁻⁴—10	0
hn ne	10⁻⁶—10⁻⁴	10— 0
ph n	×10	0
tam ne	4×10⁻	0
	4×10	◡
npoun l 48 80	◡ μg ml	0

Compound	Conc of Compound M	Per cent Inhibi tion
Hydroxylamine	5×10⁻	0
Spermine	?×10⁻⁴	0
Ethanolamine	2×10⁻⁴	1◡
	5×10⁻	3◡
	10⁻	40
Ammonium chlor de	2×10⁻⁴	10
	5×10⁻⁴	3
	10⁻³	45
Sodium borate	10⁻³	0
Sodium thiosulfate	10⁻³	0
Sodium amytal	?×10⁻⁴	10
	5×10⁻⁴	15
Sodium 5 diethyl barbiturate	10⁻³	0

TABLE Effect of various co factors

Compound	Conc of Compound M	Per cent Activation of Inhibition
Ascorbic acid	10^{-3}	0
Glutathione	10^{-3}	0
Cysteine	10^{-3}	0
Choline chloride	10^{-3}	−10
Phosphocreatine (with or without ATP)	2.5×10^{-3}	0
Pyridoxal 5 phosphate	10^{-4}	0
α Tocopheryl acetate	2×10^{-5}	0
Cocarboxylase	4×10^{-4}	0
Coenzyme A	10^{-3}	0
Riboflavin phosphate	4×10^{-4}	0
Flavin adenine dinucleotide	1.3×10^{-4}	−5
	2.6×10^{-4}	−30
β DPN (diphosphopyridine nucleotide)	1.5×10^{-3}	−10
β DPNH	1.5×10^{-3}	−25
Liver concentrate (Sigma)	200 μg/ml	−10
Yeast concentrate (Sigma)	200 μg/ml	0
DL Thioctic acid	$2 \times 10^{-5} - 10^{-4}$	0
Adenosine 3 5 phosphoric acid (with or *without ATP)*	2.5×10^{-3}	0

VII Experiment to protect or activate the uptake mechanism with co factors etc

The effect on the amine uptake of many enzymic cofactors was examined both with freshly made granules and granules kept at 0° for 24 hours since *the inactivation of the mechanism might be due to destruction of some co* factor None seemed to give a significant effect (Table 5)

Reducing agents such as ascorbic acid sulfite *glutathione* and cysteine *showed no protective influence (Tables 1 and 5)*

Isolation and storage of the granules in 0 3 M sucrose containing ethylenedi amine tetraacetate (pH 7 3 × 10^{-3} M) *did not prevent the deterioration of the* mechanism Other metal chelators (8 hydroxyquinoline penicillamine 10^{-3} M) had a slightly inhibitory action (Table 1)

The interesting work by LEHNINGER and co workers (LEHNINGER 1959 1962 LEHNINGER and REMMERT 1959 NEUBERT and LEHNINGER 1962) on

TABLE 6 Effect of sodium oleate ethyl and n butyl alcohol

The capacity of the agents to release the endogenous amines was calculated from the amine content of the test and control granules after the incubations

Compound	Conc. of Compound	Inhibition Per cent	Release Per cent
Sodium oleate	5×10^{-5} M	40	0
	10^{-4} M	65	20
	5×10^{-4} M	100	100
Ethyl alcohol	1 %	15	0
	2 5 %	25	0
	5 %	80	0–10
Butanol	0 2 %	15	0
	0 5 %	50	0–10
	1 %	90	30
	4 %	100	100

the formation of a potent uncoupling agent of oxidative phosphorylation probably a long chain fatty acid on aging of mitochondria the deleterious effects of such acids on the mitochondrial integrity and the protection with bovine serum albumin inspired the thought that a similar mechanism might operate in the granule suspensions In fact it was found that sodium oleate in low concentrations was a strong inhibitor of the amine uptake (see Section X and Table 6) However 6 per cent serum albumin in the incubation medium depressed the uptake considerably A lipid antioxidant hydroxylamine neither activated nor inhibited the amine incorporation (Table 4) Spermine effective against mitochondrial swelling was also without effect (Table 4)

When the amine granules are incubated at elevated temperatures they swell and at the same time amines and ATP are released (HILLARP and NILSON 1954 HILLARP 1958) Similarly mitochondria swell and loose their nucleotides (cf SIEKEVITZ and POTTER 1955 LEHNINGER 1962) There is a profound difference however between these two structures in respect to the effect of ATP which protects against or reverses the swelling of mitochondria apparently through a contractile mechanism (LEHNINGER 1959 1962) No such effect was observed when amine granules were incubated under the standard conditions The swelling — indicated by the decrease in light scattering at 520 mμ (cf HILLARP and NILSON 1954) — was if anything accelerated in the presence of Mg ATP

VIII Influence of adrenal cortex on amine uptake

In the course of the experiments it was repeatedly found that some samples of granules showed much higher uptake than others and that the former had been prepared from medullary tissue contaminated with adrenal cortex It was therefore thought possible that the cortex might influence the storage mechanism

In three experiments the adrenals were cut in about 2 mm thick sections From every other section the medulla was cut out with care taken to avoid contamination with cortex From the other sections it was cut out together with a small zone of the inner cortex (representing maximally 5 per cent of the tissue weight) Granule suspensions were then made in the usual way and made to contain equal concentrations of amine granules (on the basis of amine determinations) The amine uptake was compared both immediately and after storage of the suspensions at 0° for 24 hours

The contaminated granules showed a considerably higher uptake both when used fresh (33 to 65% increase) and after storage (70 to 76%) The uptake occurred only in the presence of MgATP was blocked by reserpine and thus was no unspecific adsorption

Since mitochondria are very scarce in the medulla the observed effect might be due to the increased amount of mitochondria from the cortex The mitochondrial and supernatant fractions from homogenates of adrenal cortex were therefore tested as to their effect on the amine uptake by the medullary granules The former showed a slight activation but the latter was inhibitory The mitochondrial fraction from rabbit liver (the animal was given nialamide 100 mg/kg body wt i v 3 hr before the killing) also gave only slight stimulation Liver or yeast concentrates were ineffective (Table 5)

Further experiments must obviously be made to decide whether any factor in the inner part of the cortex adjacent to the medulla is of importance for the amine storage mechanism

IX *Influence of agents affecting mitochondrial enzyme systems*

The possibility that the mitochondria present in the granule suspensions might influence the amine uptake was tested with use of inhibitors of the mitochondrial enzyme systems (*cf* LEHNINGER 1959 HUNTER *et al* 1959 CHIGA and PLAUT 1959 COOPER and KULKA 1961 WEINBACH 1961)

The results are found in Table 1 It is noteworthy that several agents showed no effect on the amine uptake in concentrations which give complete inhibition of the respiratory chain uncouple oxidative phosphorylation essentially completely block ATP exchange reactions or inhibit the mitochondrial ATPase

Several compounds producing swelling of mitochondria (i a glutathione cysteine DL-thioctic acid ascorbate arsenite sulfite thyroxine cf LEHNINGER 1959 HUNTER et al 1959 NEUBERT and LEHNINGER 1962) were not inhibitory (Tables 1 and 5)

An interesting finding is that pentachlorophenol proved to be a strong inhibitor Pentachloronitrobenzene was almost as potent while phenol and hexachlorocyclohexane (gamma isomer) showed no activity at the concentrations used

The effect of BAL and naphtoquinones which inhibit oxidative phosphorylation at the same site in mitochondria is discussed in Section XI

As stated above (Section VII) some agents with protective influence on mitochondria did not activate the amine uptake

X Some experiments on the nature of the storage mechanism

A total uptake of 10 μg catecholamines has been observed when granules (initially containing about 80 μg bound A+NA) were incubated at +37 for 30 min with an external amine concentration of 50 μg/ml If present in the intragranular water the newly stored amines thus would reach levels of about 10 000 μg/ml (calculated from data in HILLARP 1959) The storage mechanism thus is capable of accumulating amines against a high (apparent) concentration gradient (200 1) Its capacity is even more impressive in the experiments with 5 HT (see Fig 7) where the gradient must be about 1000 1

It has been proposed that the uptake and storage of 5 HT against a concentration gradient in blood platelets are due to an active transport mechanism (HUGHES SHORE and BRODIE 1958) Several features of the amine uptake in the medullary granules also suggest such a mechanism It cannot be true however that the amines are simply pumped into the granule water by a mechanism in the granule membrane External catecholamines can move rather freely to and from the intragranular water and seem to have free access to most of it (CARLSSON and HILLARP 1958 HILLARP 1959) but the labelled amines taken up are not released for days at 0

The question thus arises if they become bound in the same way as the endogenous catecholamines Since there seem to exist two different pools of amines in the granules (HILLARP 1960) two alternatives may be considered they become incorporated in the large pool of amines bound together with ATP or in the more loosely bound pool without ATP

The fact that equivalent amounts of amines and ATP are not taken up does not favour the first alternative However the final step in the uptake could

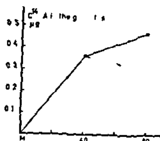


Fig 13 Uptake of C^{14}—A by the amine granules (0——0) and nondisappearance of the C^{14}—A incorporated in the granules on further incubation in a medium containing un labelled A (0 0)

Two experiments with the same sample of granules The duplicates in both experiments showed good agreement in the values obtained Glycylglycine pH 7 3 ATP 0 00 M $MgCl_2$ 0 0025 M C^{14}—A 4 3 µg/ml unlabelled A 75 µg/ml +37 0 0 at 40 min the medium was diluted seven times with a medium of the same composition but without C^{14}—A (with or without 2×10^{-6}M reserpine)

be an exchange with endogenous amines Then the uptake mechanism observed here would not be the same as that operating *in vivo* This does not seem likely

Some experiments on the stability of the labelled amine pool have been made It was not released for days if the granules were kept suspended in 0 5 M sucrose at 0° but immediately liberated on osmotic lysis If the granules (after isolation and resuspension) were reincubated at +31 in the standard medium (without C^{14} amines) with or without MgATP a slow release of both labelled and unlabelled amines occurred (10 to 15% in 30 min) The release was not markedly accelerated by reserpine (2×10^{-6} M) or tyramine (3×10^{-4} M) (*cf* EULER 1960 EULER and LISHAJKO 1960 1961 SCHÜMANN 1960) The disappearance of the labelled amines was about the same when the granules were reincubated with or without 10^{-3} M unlabelled NA in the medium Thus no rapid exchange occurs between the amines incorporated in the granules and the external amines either with or without MgATP This could be shown also in another type of experiments Granules were first incubated with C^{14} A and then diluted with the same medium containing only unlabelled A (Fig 13) Some of the C^{14} A taken up disappeared from the granules but most of the loss was accounted for by the spontaneous release occurring at elevated temperatures Another significant finding was that the loss was neither accelerated nor prevented by reserpine (2×10^{-6} M)

Thus no obvious differences in these respects were found between the labelled and endogenous amines

All findings so far on the characteristics of the uptake mechanism (MgATP dependence lability temperature coefficient blocking by drugs etc) and the

stability of the pool strongly favour the view that the mechanism is identical with that operating *in vitro* and that the amines become bound in a similar way as the endogenous amines The experiment with C^{14} ATP suggests that the amines are primarily taken up in the smaller pool of amines not containing ATP which may represent a more mobile pool Since the first direct demonstration of the existence of more than one amine pool in the adrenal medulla (HILLARP 1960) it has now become generally recognized that also other cells storing and releasing monoamines may contain such pools (*cf* SHORE 1962)

External amines can penetrate to the storage sites in the granules and there be bound by exchange with the endogenous amines (CARLSSON and HILLARP 1961) This exchange could not be blocked by reserpine (up to 10^{-4} M preincubation) and does not require an addition of MgATP Under the standard conditions used this uptake through exchange was of insignificant magnitude at external amine concentrations up to at least 6×10^{-4} M as shown by the following findings 1) Very low uptake without MgATP 2) almost complete block by reserpine 3) very slow increase in uptake when the external amine concentrations was increased above the saturation level (Fig 11) This increase may however at least partly be due to simple exchange

The finding that sodium oleate at low concentrations strongly inhibited the amine uptake might suggest that a lipid or lipoprotein is an essential component in the storage mechanism The effect of ethyl and butyl alcohol was therefore examined (Table 6) Both were strong inhibitors at low concentrations Obviously profound and unspecific structural changes may be responsible since a lipid component is of high importance for the integrity of the granules (HILLARP and NILSON 1954) The agents — especially ethyl alcohol — were inhibitory in concentrations however which did not cause an accelerated amine release It was also found that concentrations giving a strong inhibition produced no marked swelling of the granules

XI Effect of various enzyme inhibitors

Several more or less specific enzyme inhibitors were tested (Table 1) Agents found to be inhibitors were also examined as to their activity to release the granule amines in order to decide whether the inhibition was merely due to profound changes in the granule structure

Ouabain which specifically blocks active transport of cations through cell membranes in several cases (*cf* POST *et al* 1960) and also monoamine uptake by platelets and tissue slices (HUGHES SHORE and BRODIE 1958 WEISSBACH REDFIELD and TITUS 1960 DENGLER and TITUS 1961 DENGLER SPIEGEL and TITUS 1961) showed no inhibition at all in high concentration It would thus

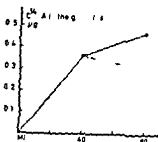


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appear that in intact monoamine storing cells two different mechanisms of amine uptake operate at the same time, i e one sensitive to ouabain and probably located in the cell membrane the other sensitive to reserpine and located in the storage granules The earlier studies on platelets lead to the concept that reserpine acts on a pump mechanism in a membrane It is not surprising that attempts (BRODIE and COSTA 1961) to explain the events in nerve endings on this basis have led to a rather confusing concept It should be emphasized that the dinstinction between the two mechanisms i e the ouabain sensitive transport mechanism and the specific reserpine sensitive storage mechanism is essential

It is noteworthy that no or but slight inhibition was caused by inhibitors of various nucleotide enzymes (ATPases transphosphorylases hexokinase) metal chelators (ethylenediamine tetraacetate inhibited only when present in concentrations enough to bind Mg^{++}) and diisopropylfluorophosphate Poly phloretin phosphate which i a inhibits several phosphatases (DICZFALUSY *et al* 1953) was active at fairly high concentrations The fact that NH reagents (acetic anhydride and phenylisocyanate but not ninhydrine) were inhibitory *to some extent may be due to an attack on the catecholamines in the medium*

Potent SH reagents give profound structural changes in the granules resulting in release of both amines and intragranular proteins (D IORIO 1957, HILLARP 1958) Mercuric chloride (10^{-5} M) and p chloromercuribenzoate (10^{-3} M) caused an essentially complete amine release and block of uptake even if the granules were exposed to them at 0 (15 min) and then were isolated and tested (Table 1) When added directly to the incubation medium mercuric chloride also at lower concentrations (10^{-6} M) gave both release and inhibition of the uptake The inhibition may thus be a consequence of structural changes

It was found however that less potent SH reagents (N ethylmaleimide allicin diethylthiosulfonate) were strong inhibitors at low concentrations although no marked amine release occurred even at higher concentrations Preincubation at 0° with allicin also largely prevented amine uptake One of the strongest inhibitors hitherto found — tranquillizers excluded — was an interesting thiosulfonate (bis 2 hydroxy benzoic acid 5 thiosulfonate kindly suggested and supplied by Dr B Hogberg AB Leo Halsingborg) This compound readily blocks SH groups e g the SH group of glutathione (tested by SH determinations according to ELLMAN 1958) Its inhibition of the amine uptake was prevented in the presence of 10^{-3} M glutathione (Table 1) This suggests an action on SH groups in the granules The compound gave a pronounced inhibition at $2 \cdot 5 \times 10^{-6}$ M but no amine release occurred even at a hundredfold higher concentration

Three other SH reagents (o iodobenzoic acid iodoacetamide sodium arsenite) showed no inhibition It is not an unusual finding however that some SH groups in enzymes do not react as easily with these reagents as with others (cf BOYER 1959) The fact that Cu^{++} and Zn^{++} (in glycylglycine buffer) had no effect may be due e g to strong binding to other groups

The finding that menadione was a fairly potent inhibitor prompted examination of other naphtoquinones and related compounds 1 4 naphtoquinone 5 hydroxy 1 4 naphtoquinone and dicoumarol were even more potent than menadione while 1 2 naphtoquinone showed about the same potency and 1 4 benzoquinone and ubiquinone were almost inactive at the same concentrations The high activity of 1 4 naphtoquinone is further seen from the fact that the granules after a short (30 min) preincubation at 0 with this compound lost their uptake capacity (Table 1)

At present no definite data are available to decide in what way the naphtoquinones interfere with the storage mechanism At the concentrations used they did not cause structural changes so profound that amine release occurred There is evidence however that 1 4 naphtoquinones easily react with free SH groups also in proteins (FIESER 1941 CAVADY and ROE 1956) In fact 2 methyl 5 8 dihydroxy 1 4 naphtoquinone has been suggested as an efficient agent for blocking SH groups (KUHN and BEINERT 1947) Evidence for the view that 1 4 naphtoquinone acts in this way was obtained in experiments with granules preincubated at 0 with this agent When such granules — after isolation — were reincubated under standard conditions the block of the amine uptake could largely be reversed by 10^{-3} M cysteine or glutathione This seems to show that some SH groups are essential for the uptake mechanism and can be blocked without profound and irreversible structural changes 1 2 naphtoquinone probably also acts on SH since this compound — like 1 4 naphtoquinone — readily reacts with glutathione (tested by SH determinations according to ELLMAN 1958)

The water soluble menadione sodium bisulfite did not inhibit the amine uptake It may not be able to penetrate to the sensitive sites or it may have lost the reactivity of menadione

BAL was found to be a fairly potent inhibitor No amine release occurred at the highest concentrations used (10^{-3} M) The experiment with preincubation at 0 showed that BAL either does not easily react at this temperature or that the blocking is reversible It may act through metal binding or reduction of S S bonds The fact that other metal chelators and agents cleaving S S bonds (sulfite cyanide) were without effect would then be due to poor penetration

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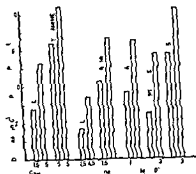


Fig 14

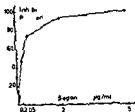


Fig 15

Fig 14 Decrease in C^{14}—A uptake on addition of L—NA tyramine tryptamine or 5—HT to the incubation medium

The same sample of granules was used in the two experiments with tyramine Left diagram the medium contained no unlabelled NA before the additions indicated Right diagram the medium contained 3×10^{-4} M NA before the additions indicated The diagram showing the effect of tryptamine and 5—HT represents the mean values from two experiments with another sample of granules The medium contained no unlabelled NA before the additions indicated The standard conditions were used for all the incubations C^{14}—A 4 μg/ml

Fig 15 Effect of Segontin on C^{14}—A uptake

Glycylglycine pH 7 3 ATP and $MgCl_2$ 0 00 5 M C^{14}—A 2 1 μg/ml total external A+NA 45 μg/ml +31 30 min

from their storage sites (unpublished data) This demonstrates the essentiality of the amino group This is further illustrated by the inhibitory action of ethanolamine and even NH_4^+ Histamine was inactive however (Table 4)

XIII Effect of miscellaneous agents interfering with adrenergic mechanisms

As reported in our previous publication a number of adrenergic blocking agents caused a blockade of the amine uptake by the granules suggesting structural similarity between binding sites of the uptake mechanism and adrenergic receptors The phenomenon has now been studied in some further detail (Table 3) Of all the α and β adrenergic blocking agents studied only

XII Effect of phenyl and indole alkylamines

Several phenyl and indole alkylamines structurally related to the catechol amines and 5 HT are able to replace the endogenous amines at the storage sites in the granules (CARLSSON and HILLARP 1961) It is thus not surprising that such compounds inhibited the catecholamine uptake Most of the tested amines (L amphetamine L ephedrine, mescaline, isopropylamino ethoxy o xylene (H 13/71) N,N diisopropyl tyramine (H 17/09) N isopropyl NA L ara mine and tryptamine) did not show a markedly stronger depression of the C^{14} amine uptake than that caused by dilution of the external amine con centration by added catecholamines No systematic study has been made however

Tyramine had the most pronounced effect of the amines tested even in concentrations which did not produce any obvious amine release (Fig 14) This is interesting in view of the finding that tyramine does not seem to be taken up through the MgATP dependent storage mechanism (Section III) although it can displace the endogenous amines in the granules Thus the possibility must be considered that the pharmacological actions of tyramine are at least partly brought about by block of the uptake mechanism

5 HT depressed the C^{14} amine uptake more and tryptamine somewhat less than catecholamines (Fig 14) The effect of 5 HT is in accordance with the high affinity of the storage mechanism for this amine (Section III) This also illustrates the importance of the 5 OH group

It is of interest that a phenoxy compound with two side chains each con taining a tertiary amino group (2 3 bis (dimethylamino ethoxy) anisole H 17/14), produced about the same inhibition as *e g* tyramine On the other hand p hydroxy phenoxyethyl trimethyl ammonium iodide (H 10/56) with a quaternary nitrogen showed no effect

Another interesting finding is that L aramine which causes depletion of tissue NA (CARLSSON and LINDQVIST 1962 UDENFRIEND and ZALTZMAN NIRENBERG 1962) showed no strong affinity for the storage mechanism In fact the decrease in C^{14} NA uptake produced by L aramine was slightly lower than that produced by L NA in the same concentration This suggests that the depletion of NA by aramine in the brain depots is due to displacement of NA (CARLSSON and LINDQVIST 1962) rather than a direct effect on the storage mechanism

Ephedrine and amphetamine were inhibitory showing that phenolic hydroxyl groups are not essential

3 4 dihydroxyphenylacetamide (H 13 49) was without effect even in very high concentrations (5×10^{-3} M) Neither can it displace the endogenous amines

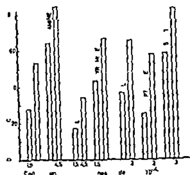


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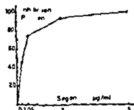


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Reserpine tetrabenazine chlorpromazine and haloperidol are all potent inhibitors (CARLSSON HILLARP and WALDECK 1962) A marked inhibition was produced by 4×10^{-8} M reserpine while isoreserpine was about 100 times weaker which demonstrates the high specificity of reserpine to act on some vital point in the storage mechanism Reserpine has been shown to inhibit the uptake of 5 HT in blood platelets *in vitro* (BRODIE *et al* 1957) and the uptake of newformed catecholamines in the granules of the adrenal medulla *in vivo* (BERTLER HILLARP and ROSENGREN 1961)

The concentrations of tetrabenazine chlorpromazine and haloperidol ne ces ary for 50 per cent block of uptake were about 5×10^{-6} 2×10^{-5} and 1 ×10 M respectively Imipramine and promethazine (Table 3) which are chemically closely related to chlorpromazine but more or less devoid of tranquillizing activity were about as potent as chlorpromazine

No amine release was caused by reserpine in concentrations much higher than those (10^{-6} M) giving an essentially complete uptake block Chlorpromazine on the other hand caused release at levels higher than 3×10^{-5} M 80 to 90 per cent were liberated at 9×10^{-5} M (+31 30 min)

The effect of reserpine was not prevented by 10^{-3} M iproniazid even if the granules were first pre incubated with iproniazid (+31 30 min) In view of the mechanism of iproniazid reserpine interaction proposed below such an effect is not to be expected Glutathione (10^{-3} M) did not influence the inhibitory effect of reserpine

The blocking effect of reserpine — unlike that of phenoxybenzamine diben

TABLE Effect of reserp ne at various concent ations of catecholam nes in tl e incuba tion me lium

The granules were incub ted under the standard conditions (+31 30 *min*) with and w th ut *reserpine* (8 10^{-8} M) Unlabelled L-NA was added to the medium to make the total n ent at on of A+NA indic ted in the table The concentration of C^{14} NA was μg ml

C ce tration f A+NA n th med um μg ml	C^{14} amine uptake μg		Inhib tion Per cent
	Controls	R serpine	
[illegible]	0 [illegible]	0 [illegible]	63
[illegible]	0 39	0 19	[illegible]1
1	0 18	0 1	33

one *i e* phentolamine proved devoid of activity on the uptake mechanism This was found to be the case also in experiments where the granules were preincubated with phentolamine

The β haloalkylamines among which phenoxybenzamine was the most potent, seemed to act by a reversible mechanism, because they were inactive when preincubated with granules for 30 minutes at 31° Thiosulfate did not interfere with their inhibitory effect which argues against the assumption that the effect is mediated via *ethyleniminium ions The mode of action of these* agents is therefore possibly not the same on the uptake mechanism as on α receptors

Also the action of 3 4 dichloro isopropylnoradrenaline (DCI) was reversible as shown by preincubation experiments

A number of vasodilating agents have been tested Of these a coronary dilating agent *i e* Segontin (N (3 phenylpropyl (2)) 1 1 diphenylpropyl (3) amine) proved most active (Fig 15) It produced a 50 per cent block in a concentration of 10^{-6} M In fact Segontin is the most potent inhibitor of the uptake mechanism known so far apart from reserpine No release occurred at a concentration giving almost complete block (6×10^{-6} M) At ten times higher concentrations a complete release took place Segontin is thus also the most active releasing agent known to date apart from mercuric chloride This enormous activity invites the speculation that a compound with similar action is involved in physiological release

Another vasodilator *i e* Nylidrine which has a chemical structure somewhat similar to that of Segontin was about ten times less potent Even less potent were papaverine, quinine and quinidine and sodium nitrite was devoid of activity

It is of interest to note that while reserpine is the strongest inhibitor of the uptake mechanism known so far a number of other presynaptic adrenergic blocking agents are devoid of such activity This is true of TM 10 bretylium tosylate and guanethidine As is well known there is evidence that these agents do not block adrenergic transmission by the same mechanism as reserpine The sites of attack of these other agents remain to be located They may possibly be sought in the *mechanism by which the transmitter is released* by nerve activity

A number of monoamine oxidase inhibitors were studied (Table 3) The two hydrazides examined were inactive (iproniazid and nialamide) The moderate activity of the other compounds may be referable to their amine and hydrazine groups

Several other compounds have been tested (see also Table 4)

XIV Tranquillizers

Reserpine tetrabenazine chlorpromazine and haloperidol are all potent inhibitors (CARLSSON HILLARP and WALDECK 1962) A marked inhibition was produced by 4×10^{-8} M reserpine while isoreserpine was about 100 times weaker which demonstrates the high specificity of reserpine to act on some vital point in the storage mechanism Reserpine has been shown to inhibit the uptake of 5 HT in blood platelets *in vitro* (BRODIE *et al* 1957) and the uptake of newformed catecholamines in the granules of the adrenal medulla *in vivo* (BERTLER HILLARP and ROSENGREN 1961)

The concentrations of tetrabenazine chlorpromazine and haloperidol necessary for 50 per cent block of uptake were about 5×10^{-6} 2×10^{-5} and $1 \cdot 5 \times 10^{-5}$ M respectively Imipramine and promethazine (Table 3) which are chemically closely related to chlorpromazine but more or less devoid of tranquillizing activity were about as potent as chlorpromazine

No amine release was caused by reserpine in concentrations much higher than those (10^{-6} M) giving an essentially complete uptake block Chlorpromazine on the other hand caused release at levels higher than 3×10^{-5} M 80 to 90 per cent were liberated at 9×10^{-5} M (+31 30 min)

The effect of reserpine was not prevented by 10^{-3} M iproniazid even if the granules were first preincubated with iproniazid (+31° 30 min) In view of the mechanism of iproniazid reserpine interaction proposed below such an effect is not to be expected Glutathione (10^{-3} M) did not influence the inhibitory effect of reserpine

The blocking effect of reserpine — unlike that of phenoxybenzamine diben

TABLE Effect of reserpine at various concentrations of catecholamines in the incubation medium

The granules were incubated under the standard conditions (+31 30 min) with and without reserpine (8 × 10 M) Unlabelled L NA was added to the medium to make the total concentration of A + NA indicated in the table The concentration of C[14] NA was μg ml

Concentration A + NA in the medium μg ml	C[14] amine uptake μg		Inhibition Per cent
	Controls	Reserpin	
3	0 8	0 9	63
1	0 30	0 19	51
	0 18	0 1	33

one i e phentolamine proved devoid of activity on the uptake mechanism This was found to be the case also in experiments where the granules were preincubated with phentolamine

The β haloalkylamines among which phenoxybenzamine was the most potent, seemed to act by a reversible mechanism because they were inactive when preincubated with granules for 30 minutes at 31° Thiosulfate did not interfere with their inhibitory effect which argues against the assumption that the effect is mediated via ethyleniminium ions The mode of action of these agents is therefore possibly not the same on the uptake mechanism as on α receptors

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SUMMARY

Bovine adrenal medullary granules were isolated and incubated with radio active adrenaline noradrenaline dopamine 5 hydroxytryptamine or tyramine The influence of various factors on the uptake of these monoamines was studied

The presence of adenosine triphosphate (ATP) was required for the uptake ATP could be replaced by some other nucleoside triphosphates but not by inorganic tripolyphosphate or adenosine di or monopho phate Experiments with C^{14} labelled ATP showed that ATP was taken up by the granules though in considerably smaller amounts than the amines when calculated on an equivalent basis In contrast to the amine incorporation the uptake of ATP was only partially inhibited by reserpine

In addition to ATP magnesium ions were essential for the uptake They could be replaced by Mn^{++} but not by Ca^{++} Several other cofactors were studied They neither protected nor activated the uptake However a factor pres nt in the inner part of the adrenal cortex appears to stimulate or stabilize the uptake mechanism

The uptake mechanism is only partially capable of discriminating between L and D adrenaline It does not discriminate between adrenaline noradrena line and dopamine 5 hydroxytryptamine is taken up by the same mechanism for which it seems to have a high affinity Tyramine is taken up by a different mechanism which does not require MgATP and is insensitive to *reserpine* but at the same time tyramine inhibits the MgATP dependent uptake mecha nism This supports the view that tyramine is taken up – at least mainly – by di placing the catecholamines from their storage sites Several other ana logues compete with the physiological amines for the uptake mechanism The amino group but not the phenolic hydroxyl groups is *essential*

The uptake mechanism operates efficiently at very low concentrations of external amines and seems to be saturated at about 4 to 5×10^{-4} M Its tem perature dependence is high

The data indicate that the amines taken up by the granules become bound in a similar way as the endogenous amines They seem to be primarily incor porated in a small labile pool not containing ATP Once incorporated they do not rapidly exchange with external amines

TABLE 8 Uptake of C^{14} ATP and C^{14} NA

0 2 ml of the granule suspension was added to 2 ml of an incubation medium containing 0 31 M glycylglycine pH 7 3 0 00[illegible] M C^{14} ATP and $MgCl_2$ 150 μg unlabelled L NA and 20 μg C^{14} NA Incubation time 30 min Reserpine 1 6 $\times 10^{-6}$ M

	Uptake of C^{14} NA μg	Total amine uptake μg	Uptake of C^{14} nucleotides μg		
			ATP	ADP	AMP
Controls 0	0 07		0 08		
Controls +31	1 [illegible]	14	1 [illegible]	0 5	0 44
Reserpine +31°	0 10	0 4	1 1		

amine and DCI — was not reversed by washing of the granules Even if the granules were preincubated at 0° (30 min) with reserpine (0 5 ml granule *suspension* 4 5 ml 0 3 M *sucrose containing* 8×10^{-8} *or* 8×10^{-7} M *reserpine*) and then isolated and resuspended the inhibition was about the same as that obtained by reserpine directly added to the medium used for testing the C^{14} amine uptake The drug thus seems to be bound to a vital site and possibly causes persistent changes in the storage mechanism even at 0°

It is of great interest that the inhibitory effect of reserpine can be counteracted by increasing the concentration of catecholamines in the incubation *medium Data from a typical experiment are found* in Table 7 Further analysis (ROSENGREN unpublished results) shows that in all probability catecholamines and reserpine compete for the same sites of the mechanism responsible for the uptake This may for the first time provide an explanation of the fact (CARLSSON *et al* 1957) that MAO inhibitors are able to prevent the depletion of catecholamine stores in tissues caused by reserpine The MAO inhibitors probably cause intracellular accumulation of catecholamines which may then counteract the effect of reserpine

The inhibitory effect of reserpine (8×10^{-8} M) could not be counteracted by increasing the Mg ATP concentration from 0 001 to 0 01 M

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A large number of enzyme inhibitors have been tested The most significant findings so far is that SH groups seem to be essential for the uptake mechanism A lipid component or nonpolar sites in a protein structure seem to be essential

A number of adrenergic blocking agents caused a blockade of the amine uptake, suggesting structural similarity between binding sites of the uptake mechanism and adrenergic receptors

Segontin (N (diphenylpropyl) amphetamine) proved to be the most potent inhibitor of the uptake mechanism known so far apart from reserpine In higher but still very low concentrations it caused complete release of the granule amines

All the major tranquillizers tested, *i e* reserpine, tetrabenazine chlorpromazine and haloperidol are potent inhibitors of the uptake mechanism Of these reserpine is by far the most potent agent (4×10^{-8} M) Isoreserpine is about 100 times weaker, indicating high structural specificity The drug seems to be bound to a vital site and possibly causes persistent changes in the storage mechanism The inhibitory effect of reserpine can be counteracted by increasing the concentration of catecholamines in the incubation medium This provides an explanation of the interaction between monoamine oxidase inhibitors and reserpine

A number of other presynaptic adrenergic blocking agents *i e* TM 10 bretylium, and guanethidine, have no influence on the uptake mechanism

Ouabain which blocks the uptake of amines and certain other cations by intact cells had no effect on the uptake of amines by the storage granule The distinction between the ouabain sensitive transport mechanism and the specific reserpine sensitive storage mechanism is essential for understanding the events at nerve endings

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From the Department of Physiology Kungl Veterinarhogskolan Stockholm and Department of Physiology University of Goteborg

Functional Organization in the Main Sensory Trigeminal Nucleus and in the Rostral Subdivision of the Nucleus of the Spinal Trigeminal Tract in the Cat

BY

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Contents

Abstract

EISENMAN, J., S. LANDGREN and D. NOVIN. *Functional organization in the main sensory trigeminal nucleus and in the rostral subdivision of the nucleus of the spinal trigeminal tract in the cat.* Acta physiol. scand. 1963. 59. Suppl. 214. — The activity of single cells in the trigeminal nucleus of the anaesthetized cat was studied. The cells were localized to morphologically defined parts of the trigeminal nucleus (*nucleus trigemini sensibilis principalis* or *nucleus tractus spinalis trigemini oralis subnucleus γ*). They were tested with different types of stimulus (mechanical, thermal, gustatory, sound, lightflash) applied to the peripheral receptors. The size and location of their receptive fields were mapped. Orthodromic latency and pattern of discharge in response to certain stimuli were recorded.

It was found that topographically organized longitudinal columns of specific secondary touch cells with small receptive fields occupied a dorsal and lateral shell of the nuclei. A column of cells discharged by periodontal receptors was located in the central region of the nuclei. Cells responding to noxious stimuli were mainly found in the central and medial parts. The results indicate a longitudinal distribution of the cells assumed to subserve tactile perception. They do not support the theory of GERHARD (1923) and SJÖQVIST (1938) according to which the tactile relays are restricted to the main sensory nucleus. The majority of the cells in the longitudinal columns are discharged only by one of the different types of stimuli applied and they are therefore specific in the sense of JOHANNES MÜLLER.

Methods

Anaesthesia and surgical procedure

All experiments were done with cats anaesthetized with pentobarbitone sodium in an initial intraperitoneal dose of 40 mg/kg and subsequent intravenous doses of 5—10 mg administered when required to maintain the anaesthesia.

The dorsal aspect of the cerebellum was exposed by a hole in the skull to the right of the midline just behind the bony tentorium. Parts of the tentorium was also removed in experiments on the rostral end of the main sensory nucleus. The cat's head was fixed in a Horsley Clarke apparatus. The bars of the head holder used for the fixation of the upper jaw were modified to give as free access as possible to the right half of the face and to the mouth (GORDON, LANDGREN and SEED 1961). The cervical vertebral column was fixed by two rods pressing against the two sides of the C_3—C_4 region. The recording microelectrodes were placed in the trigeminal nucleus by means of Horsley Clarke readings in a system with the vertical axis at 30° angle to the conventional one, which made the penetrations through the cerebellum approximately parallel to the tentorium. Leaving the cerebellum intact protected the underlying medulla, reduced interferences with its blood supply and reduced disturbing movements due to pulsations of the brain.

Recording technique

Unitary responses were recorded extracellularly by means of glass pipette microelectrodes (tip diameter about 1 μ) filled with 4 3 molar NaCl solution or by tungsten or steel micro-electrodes pointed by electropolishing and insulated according to a technique modified from HUBEL (1957). In our hands the glass pipette microelectrodes gave a higher yield of single units than the metal electrodes and the latter were therefore mainly used to relate the site of a response to an identifiable lesion in the series of histological sections. The action potentials were recorded by a conventional system consisting of a cathode follower, a RC-coupled amplifier, a double beam CRO and a Grass camera.

Stimulation technique

Two Grass stimulators with stimulus isolation units were used for the electrical stimulation of the receptive field. The stimulus was applied via a pair of silver wires touching the moistened surface of the skin or mucous membrane. Occasionally steel needles inserted into the skin were used as stimulating electrodes.

The following physiological stimuli were used: Light touch with a soft brush or a small glass rod, pressure with a glass rod, pinpricks and pinching with a

Introduction

Recent investigations into the organization of the trigeminal nucleus (GORDON, LANDGREN and SEED 1960 and 1961, KRUGER, SIMINOFF and WITOWSKI 1961, WALL and TAUB 1962) all agree in their observation that the nucleus of the spinal trigeminal tract shows the features of a typical tactile relay nucleus. Cells responding with short latency to light touch of hairs or skin within well defined and constant receptive fields are thus arranged in such a way within this nucleus that they form a detailed topographical map of the body surface. This result is somewhat surprising in view of GERHARD's (1923) and SJÖQVIST's (1938, 1939) generally accepted interpretation that the main sensory trigeminal nucleus is the relay of the tactile fibres from the face and that fibres subserving pain and thermal sensations are relayed in the nucleus of the spinal trigeminal tract.

WALL and TAUB (1962) also investigated the main sensory nucleus and found that it was characterized by cells with large receptive fields located mainly on the outer surface of the ipsilateral face. A variety of afferent fibre types converged onto these cells and the topographical organization was less well maintained as compared to the middle and rostral parts of the nucleus of the spinal trigeminal tract. These findings further emphasize the divergence between the important results of GERHARD and of SJÖQVIST and those building on detailed analysis of single unit responses within the trigeminal nucleus. Because of the importance of this conflict for our understanding of the functional organization of the trigeminal nucleus it was considered valuable to sample further detailed information from single cells within the main sensory trigeminal nucleus and the rostral part of the nucleus of the spinal trigeminal tract. In the present series particular attention was paid to the problem of localizing the observed units to morphologically described parts of the nucleus.

The cortical and thalamic neurones of the trigeminal path were studied by LANDGREN (1957 a, b, 1960 a, b) in a number of previous investigations. A particular point was made of applying different types of stimulus to the receptive field of the neurones. This line was followed also in the present experiments dealing with the medullary link in the trigeminal path. Further evidence of specificity of the trigeminal paths in the sense of JOHANNES MÜLLER (1835) will be presented in this report. The ascending projections of secondary trigeminal neurones will be described in a subsequent publication (EISENMAN, FROMM, LANDGREN and NOVIN 1964).

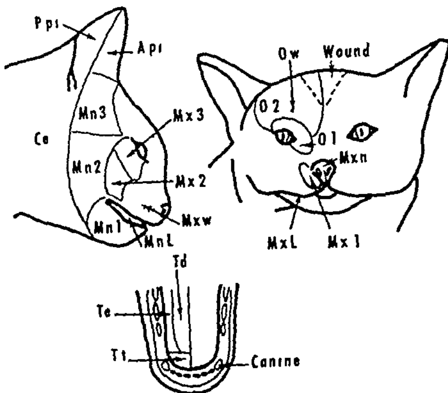


Fig 1 Code used to describe receptive fields of units Mn Mx O=Mandibular Maxillary and Ophthalmic dermatomes respectively T=tongue W=whiskers L=lip

variations which is too large to permit the localization of recording positions in different animals to a certain part of a subnucleus

The following method was used to relate recording positions along a micro electrode track to the histological section In each experiment certain rostrocaudal levels separated by at least 1 mm were chosen for investigation At such a level a transverse grid of parallel recording tracks was made across the nucleus using only one glass pipette microelectrode for all the tracks The microelectrode was cut and left in the last track until after fixation At the end of the experiment a tungsten or a steel microelectrode was chosen and its length was related to that of the glass pipette microelectrode by means of a measuring microscope with scale readings down to 0 01 mm A track was made through the nucleus with this electrode observed units were analyzed and the coordinates of the upper and lower borders of the tactile responses from the face were noted as well as the coordinates of the maximum amplitude focal potential evoked by electrical stimulation of the tip of the tongue Finally two electrolytic lesions (HUBEL 1959) were made at the lower and upper borders of the responding region The coordinates of the lesions were recorded When steel electrodes were

pair of forceps An electrically controlled mechanical stimulator (GORDON and PAINE 1960) was sometimes used for repetitive mechanical stimulation of the receptive field

Radiant heat ice and in some experiments an ethyl chloride spray were used as crude thermal stimuli When applied for a sufficient time these stimuli may be noxious Units responding to them were therefore tested with the thermode technique of HENSEL STRÖM and ZOTTERMAN (1951) With this method rapid changes between constant temperatures can be applied to the skin or the mucous membranes A series of seven water baths supplied a distribution panel with seven stopcocks The water was allowed to flow continuously through the system and was bypassing the thermode when this was not in use Water of seven constant temperatures were therefore always available for the thermode The temperatures used were 10 28 35 37 40 46 and 55°C The thermode was always cooled or warmed from a control level of 37°C The temperature of the water was measured with thermocouples at the outflow near the thermode and recorded on an automatic compensator with 12 recording channels (Philips PR 3210 A/00) The temperature of the silver plate of the thermode was measured with a thermistor and recorded on one of the beams of the CRO This method allowed us to carry out a quantitative analysis of the response to thermal stimuli within the limited time available for the study of a single cell

Units responding to stimulation of the tongue were tested with taste solutions i e 0 5 M NaCl 0 01 M Quinine HCl in RINGER s solution 0 3 M acetic acid in RINGER s solution (PH 3) and distilled water

In addition light flashes auditory stimuli and vibrations produced by tapping of the stand were routinely presented to reveal convergence of a type expected to be found on cells in the reticular formation

Description of the receptive field

The location and extent of the receptive field was described in terms of the subdivisions of the face and mouth drawn on the outline maps shown in Fig 1 A code was developed to name the subdivisions In this code subdivisions of the ophthalmic dermatome are *designated with an O the maxillary with Mx* and the mandibular dermatome with Mn W stands for whisker L for lip and T for tongue and Pi for pinna Where a receptive *field could not be adequately* described in terms of the code it was drawn on a standard outline drawing of the face

The localization of the recorded responses

The method we have *chosen for our attempts to describe the anatomical* location of our responses rests on the cytoarchitectural definitions of the trigeminal subnuclei In order to *facilitate the comparison* between the present results and those of other authors using obex as a reference point the borders of the subnuclei are *related to obex in* the anatomical section of this paper *In our experi*ence the use of obex as a reference point introduces an error due to individual

mediolateral and rostrocaudal direction the error should be less as the localization in these directions depends directly upon the traces left by the recording microelectrode in the histological section

In the present series 50 maps of the type described above were prepared from grids of tracks passing *Nucleus trigemini sensibilis principalis* or *Nucleus tractus spinalis trigemini oralis* γ in 20 experimental animals

used iron was similarly deposited in two spots by passing a current of 1 micro ampere through the microelectrode for 10 seconds India ink tracks were then made at known distances apart and also at known distance from the level of the grid as described by GORDON *et al* 1961

The cat's head was then perfused with 0 9 % NaCl followed by BODIAN's solution When steel electrodes had been used for marking 3 % potassium ferro cyanide in 10 % formaline solution was used for the perfusion After fixation the skull was opened and the head replaced in the Horsley Clarke apparatus The brain still in situ was then blocked with a knife carried by the micro manipulator in planes approximately parallel to that of the microelectrodes The blocks were embedded in celloidin and serial sections (50 μ thick) were cut using the india ink tracks for orientation The sections were stained with toluidin blue or else alternate sections were stained with toluidin blue and with LOYEZ fibre stain

When examining the sections it was easy to identify the blue spots made by the steel electrodes but somewhat more difficult to find the electrolytic lesions left by the tungsten electrodes It was also easy to follow the full length of the last track left by the cut glass pipette microelectrode (See track VI in Fig 5) The other tracks could generally be seen only when passing through the cere bellum as only the coarse shaft of the electrode left traces visible in the sec tions As these tracks were made with the same microelectrode they were how ever assumed to run parallel to the last easily identified track and this assump tion was confirmed by occasional traces observed further down the tracks (Fig 5 B) The section showing the passage through the trigeminal nucleus was projected onto paper and a 20 times enlarged tracing was made All traces left by the microelectrodes in the different sections were projected and traced onto this map as shown in Fig 5 A Lines were drawn along the traces left by identi fied tracks down through the trigeminal nucleus A 60 times enlarged map of this nucleus and its immediate surroundings was then prepared (Fig 5 C) show ing the course of the tracks

Shrinkage which is a factor of great variability was estimated from the measured distances between the electrolytic lesions or blue spots (Fig 5 B large curved arrows) The vertical coordinates of the observations made with the glass pipette microelectrodes were related to the blue spots or the lesions by means of the above mentioned measurements of the length of the two electrodes Allowing for shrinkage the positions of the observations were finally laid out on the map (as shown in Fig 5 C) The agreement in location of the responses recorded by the two electrodes demonstrated a successful reconstruction

The error involved in the localization of our recording positions according to the above described method depends on the following factors

a) The error in reading the micrometer scales (backlash etc)
b) The actual position of the microelectrode tip relative to the centre of the marking spot
c) The shrinkage of the tissue due to the histological procedure
d) The dragging of the tissue along with the microelectrode

Considering these factors we appreciate the error in our localization in dorso ventral direction (along the recording track) to be between 0 1 and 0 2 mm In

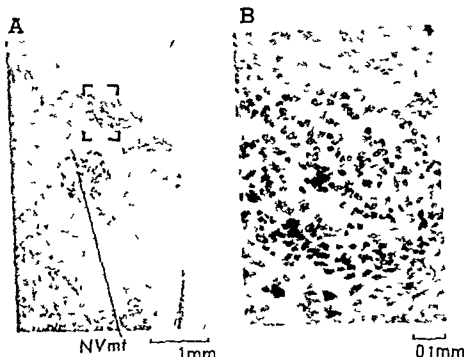


Fig 2

A Transverse section through rostral pole of NVsnpr
B Region enclosed by brackets in A at higher magnification

Bech — Nucleus Bechterew
Dt = Nucleus Deiters
NVmt = Nucleus trigemini motorius
NVsnpr = Nucleus trigemini sensibilis principalis
NVspoα = Nucleus tractus spinalis trigemini oralis subnucleus α
NVspoβ = Nucleus tractus spinalis trigemini oralis subnucleus β
NVspoγ = Nucleus tractus spinalis trigemini oralis subnucleus γ
NVII = Nucleus nervi facialis
Ols = Nucleus olivaris superior
V = Nervus or tractus spinalis trigeminus
VII = Nervus facialis
30 μ celloidin sections stained with Toluidine Blue

scattered among small neurones of irregular size and shape The cell density is considerably lower than that of NVsnpr We have found that these criteria could be applied to the cat (Fig 4 A and C)

A few similar large neurones may be found in the caudal part of NVsnpr which overlaps with subnucleus γ They are however always much more common in the last mentioned nucleus The large neurones in NVspoγ appeared to

Results

I Anatomy

We have attempted to study certain functional characteristics of the neurones in the trigeminal nucleus of the cat and to localize the investigated neurones to morphologically defined parts of the nucleus. As no detailed investigation of the cat's trigeminal nucleus seems to exist we decided to build on MEESSEN and OLSZEWSKI's (1949) description of the same nucleus in the rabbit. In our experience there is a good agreement between the cytoarchitecture of the trigeminal nuclei in these two animals, as will appear if the following summary of our morphological observations is compared with the description given by MEESSEN and OLSZEWSKI (1949).

Nucleus trigemini sensibilis principalis (NVsnpr)

The main sensory nucleus is a rather compact nucleus of rounded cells uniform in size and shape (Fig. 2 B and Fig. 3 D). In our sections they appeared to have a diameter of 15—30 μ. Their size was about 1/3 to 1/2 of that of the cells in *Nucleus trigemini motorius* (NVmt). The rostral border of NVsnpr roughly corresponds to that of NVmt. At the rostral pole the dorsomedial part of NVsnpr generally forms a round island of densely arranged cells extending about 50—100 μ rostrally to NVmt (Fig. 2 A and B). The sensory and motor nuclei are separated by a zone containing only few cells (cf. regio II of MEESSEN and OLSZEWSKI).

The caudal end of NVsnpr is found up to 1400 μ caudal to NVmt, where the fibres of the VIIth nerve travel across the medulla. Caudally to NVmt it overlaps with *Nucleus tractus spinalis trigemini oralis* γ lying laterally between this nucleus and the spinal trigeminal tract (Fig. 3 B). Dorsally NVsnpr is related to *Nucleus Bechterew*. *Nuclei olivaris* and *paraolivaris superior* are found just ventral to its caudal half.

NVsnpr has a rostrocaudal extent of about 2.5 mm and is found approximately 6—10 mm rostral to obex and 3.5—5.5 mm lateral to the midline.

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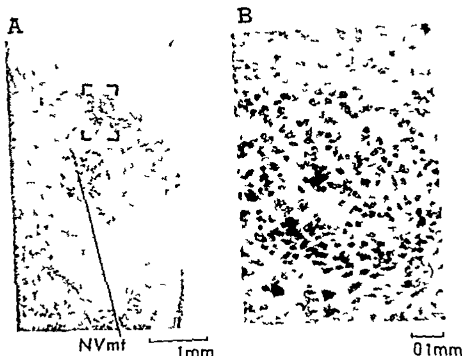


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The caudal end of NVsnpr is found up to 1400 μ caudal to NVmt where the fibres of the VIIth nerve travel across the medulla. Caudally to NVmt it overlaps with *Nucleus tractus spinalis trigemini oralis γ* lying laterally between this nucleus and the spinal trigeminal tract (Fig. 3 B). Dorsally NVsnpr is related to *Nucleus Bechterew*. *Nuclei olivaris* and *paraolivaris superior* are found just ventral to its caudal half.

NVsnpr has a rostrocaudal extent of about 2.5 mm and is found approximately 6—10 mm rostral to obex and 3.5—5.5 mm lateral to the midline.

Nucleus tractus spinalis trigemini oralis γ (NVspoγ)

This subnucleus forms the rostral division of the nucleus of the spinal trigeminal tract. According to MEESSEN and OLSZEWSKI it is characterized by large neurones

have a diameter of 40—60 μ. They have large dendrites and are sometimes almost as large as the motoneurones in NVmt (cf Fig 3 C and Fig 4 C). The majority of the cells in subnucleus γ are small and have a diameter of about 10—25 μ.

Subnucleus γ is found in the region between NVmt and *Nucleus nervi facialis* (NVII). Its rostral end generally appears about 300 μ caudal to NVmt and some 100 μ rostral to NVII. It is replaced by subnucleus β. A short region of overlap between γ and β is sometimes seen with subnucleus γ lateral to β.

The rostrocaudal extent of NVspoγ is about 1.5 mm, the region of NVsnpr and NVspoγ overlap being roughly 1 mm. It is found approximately 5—6 mm rostral to obex and 3.0—5.5 mm lateral to the midline.

Nucleus tractus spinalis trigemini oralis β (NVspoβ)

Subnucleus β differs from subnucleus γ in its lack of large neurones and in having a more cell rich cytoarchitecture (cf Fig 4 C and D). In our sections the cell diameters varied from 10 to 40 μ. The shape of these neurones is not as uniform as that seen in NVsnpr, nor is the density of subnucleus β comparable with that seen in the main sensory nucleus.

The rostrocaudal extent of subnucleus β corresponds approximately to that of NVII. However, in cat a pure β type of nucleus is only seen at the rostral pole of NVII. At the level of the caudal part of this nucleus large neurones appear laterally near the spinal trigeminal tract indicating an overlap between subnuclei β and α.

The length of NVspoβ is about 1 mm. It is found approximately 4 to 5 mm rostral to obex and 2.5 to 5.0 mm lateral to the midline.

Nucleus tractus spinalis trigemini oralis α (NVspoα)

This subnucleus has large neurones with diameters of 40—90 μ scattered among smaller cells (10—30 μ). It is found at the level of *Nucleus olivaris principalis* and extends roughly from the level of obex to NVII. At the caudal end of NVII it overlaps on the lateral side of subnucleus β.

At the level of obex NVspoα is replaced by *Nucleus tractus spinalis trigemini caudalis* (NVspc). The three subnuclei (*magnocellularis*, *gelatinosus* and *zonalis*) do not appear clearly until about 0.5 mm caudal to obex.

Fig 3

A. Transverse section through NVsnpr at the level of NVmt.

B. Zone of overlap of NVsnpr and NVspoγ caudal to NVmt.

Note the greater cell density in NVsnpr and the characteristically large neurones in NVspoγ.

C. Region of NVmt enclosed in brackets in A at higher magnification.

D. Region of NVsnpr enclosed in brackets in A at higher magnification.

1 mm scale refers to A and B, 0.1 mm scale to C and D.



A
Bech
NVsnpr
V
C
NVmt
1mm



B
NVspo
NVsnpr
Dt
V
Ols
D
VII

0 1mm

have a diameter of 40—60 μ They have large dendrites and are sometimes almost as large as the motoneurones in NVmt (cf Fig 3 C and Fig 4 C) The majority of the cells in subnucleus γ are small and have a diameter of about 10—25 μ

Subnucleus γ is found in the region between NVmt and *Nucleus nervi facialis* (N VII) Its rostral end generally appears about 300 μ caudal to NVmt and some 100 μ rostral to N VII it is replaced by subnucleus β A short region of overlap between γ and β is sometimes seen with subnucleus γ lateral to β

The rostrocaudal extent of NVspoγ is about 1 5 mm the region of NVsnpr and NVspoγ overlap being roughly 1 mm It is found approximately 5—6 mm rostral to obex and 3 0—5 5 mm lateral to the midline

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The rostrocaudal extent of subnucleus β corresponds approximately to that of N VII However in cat a pure β type of nucleus is only seen at the rostral pole of N VII At the level of the caudal part of this nucleus large neurones appear laterally near the spinal trigeminal tract indicating an overlap between subnuclei β and α

The length of NVspoβ is about 1 mm It is found approximately 4 to 5 mm rostral to obex and 2 5 to 5 0 mm lateral to the midline

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At the level of obex NVspoα is replaced by *Nucleus tractus spinalis trigemini caudalis* (NVspc) The three subnuclei (*magnocellularis gelatinosus* and *zonalis*) do not appear clearly until about 0 5 mm caudal to obex

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Note the greater cell density in NVsnpr and the characteristically large neurones in NVspoγ

C Region of NVmt enclosed in brackets in A at higher magnification

D Region of NVsnpr enclosed in brackets in A at higher magnification

1 mm scale refers to A and B 0 1 mm scale to C and D

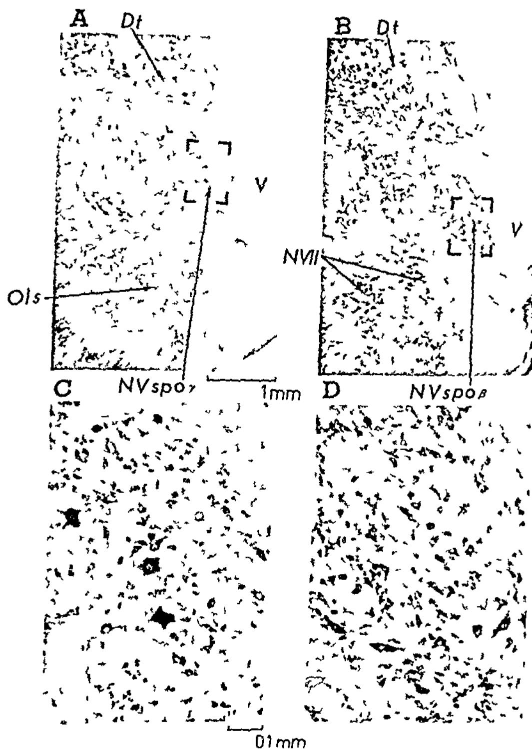


Fig. [illegible]

A Transverse section through [illegible] caudal [illegible] NVsopr

B Transverse section through NVspoβ at [illegible] [illegible] NVII

II The functional organization of NVsnpr and NVspov

The following considerations are based upon records from single trigeminal neurones In addition responses from several units were recorded These responses helped in localizing the activated region and filled out the gaps between the observed units However their origin cannot be precisely localized and they were therefore not considered in the final analysis

The division of the units into primary and higher order neurones

The first treatment of the responses from the trigeminal units was a division into those obtained from primary neurones i e from afferent fibres and those obtained from secondary or higher order neurones within the nucleus We have no theoretical basis for making this division but we have used an empirical method involving the following criteria (cf GORDON *et al* 1961)

1 Units found in tracks which upon histological examination were clearly localized to the fifth tract were excluded
2 Units showing a monophasic positive spike and no spontaneous activity were excluded

The rest of the units localized to the trigeminal nucleus were assumed to be secondary or higher order neurones and they will be referred to as cells

An attempt was made to apply still more rigorous criteria for the selection of secondary or higher order neurones For a unit to be accepted in this group it was thus required that it should respond to antidromic stimulation of the ascending trigeminal paths or show a transsynaptic response to such stimuli Units with receptive fields exceeding the dermatome of one of the three trigeminal branches were also accepted During the analysis cells fulfilling these requirements were marked with an asterisk and the group will be referred to as starred cells

It may be necessary to explain why units with monophasic positive spikes and without spontaneous activity were excluded from the cell group The reasons were as follows Units localized to a cell free region of the trigeminal tract showed a monophasic positive spike in combination with zero spontaneous activity in 84 % of the cases (17 units of 20) In the group of starred cells however these features were observed only in 3 4 % (4 units of 116) As the combina

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C Bracketed region in A at higher magnification
D Bracketed region in B at higher magnification
Note the large polygonal neurones characteristic of subnucleus γ (C) and the greater cell density in subnucleus β (D)

of a monophasic positive spike and no spontaneous activity was common ons and rare in nuclear neurones it was thought that removal of such units d tend to eliminate primary afferent fibres from our data TASAKI POLLEY ORRECO (1954) have previously made similar observations and used them lassification of presynaptic responses in the geniculate body

Spontaneous activity and spike shape

present observations were made on 246 cells localized to NVsnpr ells localized to NVspo, and 8 cells found in the border zone between e nuclei Out of these 340 cells, 131 belonged to the group of starred defined above

ontaneous activity was seen in 57 % of the cells When the starred were considered alone this percentage was found to be 64 The spike monophasic positive in 6 % of the cells and negative or diphasic in o 74 % of the cells showed high frequency injury discharge when were finally lost

ontaneous activity was found only in 16 % of the units anatomically ized to the trigeminal tract The spikes of these units were mono ic positive in 84 % of the observations and they never showed an y discharge

The classification of the trigeminal cells

he *type of stimulus* effective in discharging the cell was used as primary criterion for the classification The following groups were ted

ells responding to light mechanical stimulation of the receptive field ich as light touch with a blunt glass rod (2 mm diameter at the end sed for touching) or a soft brush These types of stimuli will be eferred to as touch and the cells in this class will be called touch cells

ells responding to mechanical stimulation of a moderate strength ich as moderate pressure with the glass rod They will be referred as pressure cells

ells responding to strong mechanical stimuli such as pinching with orceps or pinprick and cells responding to heating above +50°C or cooling below +10°C These types of stimuli will be referred to as oxious and the cells will be called nociceptive cells

ells responding to gentle tapping of the teeth or to pressure on the ngiva on both sides of the alveolar process These cells were always

tested by tapping the stand to separate them from cells responding to general vibration of the skull and to sound a type that was regularly found in *nucleus olivaris superior* ventral to the trigeminal nucleus

5) Cells responding to movements of the jaw

B The second criterion used was *the size of the receptive field* According to this criterion two groups were found

1) Cells with well defined stable receptive fields restricted to parts of the dermatome of one of *the three trigeminal branches This type of* receptive field will be referred to as *small*

   This group was formed by the pooling of three subgroups used during the investigation These were

   a) very small receptive fields with a diameter of about 5 mm or receptive fields consisting of one or two vibrissae or a few hairs

   b) Receptive fields of the order of 1 cm restricted to a subdivision of the dermatomes as described in Fig 1 Receptive fields of more than two vibrissae also belonged to this subgroup

   c) Receptive fields extending beyond the borders of our subdivisions of the dermatome although not beyond the borders of this dermatome

2) Cells with receptive fields exceeding the borders of the dermatome of one of the trigeminal branches These cells will be referred to as *wide receptive*

C The *location* of the receptive field was used as the third criterion During the experiment the location was recorded as described in methods (Fig 1) When analyzing the material it was found convenient *to divide the* receptive fields into three main groups

1) Oral (Tongue floor of the mouth gingiva on the alveolar processes hard palate inside of lips teeth)

2) Perioral (Lips nose Mnl MxL Mxw Mx2 Mxn according *to Fig 1)*

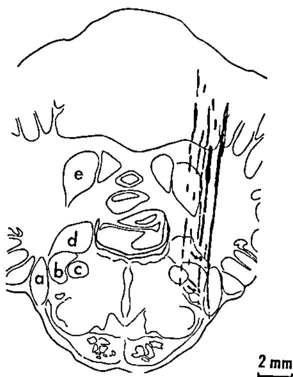
3) Periorbital (Eyelids nictitating membrane and O1 Ow Mx3 according to Fig 1)

In addition to this a separate record was kept of receptive fields including the cornea The vibrissae were also *studied as a separate group*

The localization of the cells within the trigeminal *nucleus*

In order to describe the data underlying the following analysis the results of one experiment are shown in Fig 5 In this experiment a row of recording tracks was made through N V snpr The reconstruction was done as described in methods All tracks were identified and traced (Fig

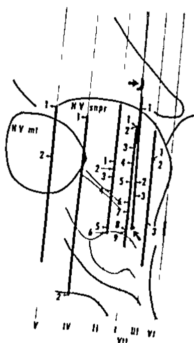
A



B



C



5 A and C) It was possible to follow some of them into VI snpr as seen in the photomicrograph of Fig 5 B Responses to physiological stimulation of the face or mouth were obtained along those parts of the tracks marked as heavy bars in Fig 5 C There is obviously a good agreement between the borders of the responsive region and the borders of the trigeminal nucleus derived from the histological section

The recorded cells were localized (figures near tracks in Fig 5 C) on a tracing of the section from which the photomicrograph of Fig 5 B was taken The effective stimuli and the location of the receptive field of the cells are listed below Unless specially noted the receptive fields were strictly ipsilateral

Track I cell *1* Responded to light pressure inside and near the angle of the mouth *2* Touch Te middle third *3* and *4* Touch hairs Mnt *5* and *6* Touch MxL *7* and *8* Touch Mxn *9* Touch hairs O2

Track II cell *1* Pressure Mn? *2* Tapping of Mx teeth *3* Tapping of Mx incisors *4* Pressure MxL near philtrum *5* Touch Mxn *6* Touch hairs O1 and O?

Track III cells *1* and *2* Touch hairs Mx2 near the angle of the mouth *3* Touch far back in the mouth

Track IV cell *1* Touch Te *2* Pressure and pinprick all face (Mn Mx O bilateral) No response to noxious mechanical stimulation of the paws or body nor to light flash or sound No response to noxious thermal stimuli applied to eye and nose

Track V cell *1* Responds to opening of the mouth *2* Pressure Te middle third

Track VII cell *1* Touch hairs Mn? *2* Touch hairs O1 edges of eyelids No response from the cornea *3* Touch hairs O1 edges of eyelids and cornea

The maps showing the localization of the cells were arranged according to their rostro caudal positions and compared The caudal pole of NVmt was used as a reference point when the positions were determined

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Fig Results from one experiment at the level of NV snpr

A Outline drawing of transverse section at the level of penetrations through NV snpr Superimposed on this are the traces left by microelectrodes in neighbouring sections The arrows point to the two Prussian Blue reference spots made in track VII a=root of V 1=NV snpr c=NVmt d=Bech e=Nucleus interpositus

B Photomicrograph showing traces left by penetrations through NV snpr (small horizontal arrows) and Prussian Blue reference spots (large curved arrows)

C Outline drawing of B Responses from the face were obtained from those parts of the tracks marked by heavy bars The numbered arrows indicate the location of single units described in detail in the text The Roman numerals indicate microelectrode penetrations in the order of their placement

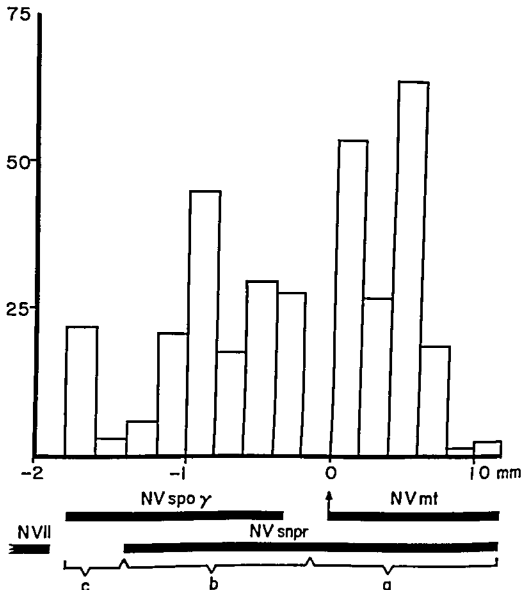


Fig 6 Diagram showing the rostro caudal distribution of the investigated trigeminal cells The caudal pole of NVmt was used as a reference point The cells in segments a b and c were plotted on standard diagrams representative of each segment (cf Fig 7 9)

NVsnpr and NVspoγ were divided into three transverse segments These were a) NVsnpr at the level of NVmt b) the region of overlap between NVsnpr and NVspoγ and c) NVspoγ caudal to NVsnpr The borders of the trigeminal nucleus and of neighbouring structures were comparable within these segments A tracing of a section selected from the middle

of the segment was taken to represent the topography of the segment and used as a standard diagram upon which all cells found in the segment were plotted in positions approximately corresponding to their location on the original maps. Such standard diagrams are shown in Figs. 7—9. The rostro-caudal distribution of the cells within the three segments is given in Fig. 6.

For special purposes segment a) was split in two divisions by separating out the cells found 0—199 μ rostral to the caudal pole of NVmt and plotting them on a standard diagram from this region (Figs. 8 D and 9 B). This was done in order to illustrate more correctly the localization of cells in the immediate surroundings of NVmt.

Touch cells with small receptive fields

A. Distribution within the nucleus and location of receptive fields

At the level of NVmt this group of cells occupies a dorsal and lateral position within NVsnpr. As shown in Fig. 7 A and B, there is a clearcut topographical organization within this part of the nucleus. The touch cells connected to the tongue are marked with dots. They form a column of cells situated dorsally in NVsnpr. Dorsolaterally in the nucleus there is a column of touch cells with perioral receptive fields. Those responding to touch of the mandibular dermatome (Fig. 7 B, ○) lie dorsal to the maxillary touch cells (△). In the ventrolateral part of the nucleus periorbital responses were obtained (□). It is obvious from Fig. 7 A and B that the touch cells are rare in the central and medial parts of NVsnpr.

The distribution of the touch cells with small receptive fields within the region of overlap between NVsnpr and NVspoγ is shown in Fig. 7 C and D. The same topographical organization is seen as in Fig. 7 A and B. Columns of cells with oral, perioral mandibular, perioral maxillary and periorbital receptive fields are thus found dorsally and laterally in this segment also. The perioral and periorbital columns fill out NVsnpr and the oral column is found dorsally in NVspoγ. The columns continue into NVspoγ caudal to NVsnpr as indicated by Fig. 8 C. The topographical organization of the touch cells with small receptive fields is thus independent of the great difference in cytoarchitecture between the two subnuclei.

In Fig. 7 C the tongue column (dots) seems to be split in two divisions by periodontal cells responding to gentle tapping of the teeth (Λ). This separation may not be significant. It could very well be due to the small

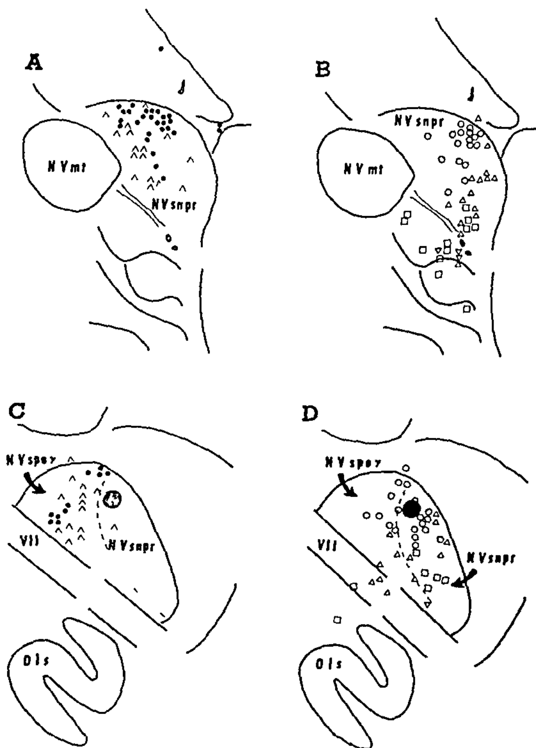


Fig 7 Diagram summarizing the distribution of touch cells with small receptive fields and cells with periodontal receptive fields

number of our observations and the errors involved in our localization method

As shown in Fig 7 D several touch cells with small perioral or periorbital receptive fields were localized to Vspoy in a region near its border with Vsnpr These symbols may represent responses from axons of touch cells on their way from Vsnpr to the contralateral trigeminal lemniscus It can however not be excluded that they represent cell bodies actually located in Vspoy

A detailed study of the topography revealed that touch cells with receptive fields on the mucous membranes of the floor of the mouth the hard palate and the gingiva were localized to a region just ventral to the tongue nucleus These cells lie in the border zone between the tongue region and the centrally situated nucleus of periodontal responses (Fig 7 A ∧)

Cells responding to touch of the naked part of the nose around the nostril were found among the periorbital touch cells ventrolaterally in Vsnpr They were thus separated from the group of cells with receptive fields within the maxillary dermatome In Fig 7 B they are marked ▽ This localization would be expected if the cat's ophthalmic dermatome also includes the tip of the nose as is the case in man (cf DEJERINE 1914)

Touch cells with a receptive field including the cornea were often found in Vsnpr at the level of Vmt They were generally found among the periorbital cells in the ventrolateral part of the nucleus but the cornea region seemed to extend further medially For example two cells responding only to touch of the cornea were isolated in a nucleus of similar responses found in the central part of Vsnpr somewhat lateral and ventral to Vmt (See Fig 8 D)

According to DEJERINE (1914) ascending syringobulbia causes a sensory deficit starting in a region involving parts of all the three trigeminal dermatomes but located far away from the mouth As the process ad

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B Cells with perioral and periorbital receptive fields found in Vsnpr at the level of Vmt

C Cells with oral receptive fields found in region of overlap of Vsnpr and Vspoy

D Cells with perioral and periorbital receptive fields found in region of overlap of Vsnpr and Vspoy

(●) = tongue receptive field

(○) = perioral mandibular receptive field

(△) = perioral maxillary receptive field

(□) = periorbital receptive field

(▽) = receptive field on naked tip of nose

(∧) = periodontal receptive field

Shaded areas in diagrams are reference spots

vances rostrally in the medulla, sensation is lost in concentric zones more and more approaching the mouth. The observation suggests a rostro-caudal organization of the trigeminal nucleus with perioral reception located near the rostral pole. The existence of a column of periorbital touch cells extending to the rostral part of NVsnpr is at variance with the observations of DEJERINE. It was found, however, that the majority of the perioral touch cells in the rostral half of NVsnpr (13 out of 21 cells) had their receptive fields on the lips, whereas the majority of these cells found in the caudal half had receptive fields localized to the hairy skin surrounding the mouth (32 out of 39 cells). It is also of interest to note that touch cells with receptive fields on the cheek (Mn2, cheek w) or near the anterior base of the pinna (Mn3, O2) were very rare both in NVsnpr and in NVspoy. In all, 8 such cells were recorded, being only 5 % of the total material of touch cells with small receptive fields.

Table I. The location of the receptive fields of touch cells with small receptive fields

NVsnpr

Oral		Perioral				Periorbital	
		Mandibular		Maxillary			
T	34 cells	MnL	12 cells	MxL	7 cells	O1	13 cells
Gi	4	MnL Mn1	3	MxL Mxn	1 cell	Cornea	2
Floor		Mn1	17	Mxn	4 cells	Cornea O1	2
Pal				Mx1	12	Mx3	1 cell
				Mx2	2		
	38 cells		32 cells		26 cells		18 cells

Total NVsnpr=114 cells

NVspoy

Oral		Perioral				Periorbital	
		Mandibular		Maxillary			
T	10 cells	MnL	4 cells	MxL	1 cell	O1 Ni	1 cell
T MnL	2	MnL Mn1	1 cell	Mx1	3 cells	O1 Ow	1
Pal	1 cell	Mn1	2 cells	Mx1 Mxn	1 cell		
				Mx1 Mxw	1		
	13 cells		7 cells		6 cells		2 cell

Total NVspoy=28 cells

Total overall=142 cells

[1] For interpretation of the code of the receptive fields see Fig. 1. Gi=gingiva, Floor [illegible] mucosa, Pal=hard palate, Ni=nictitating membrane

Touch cells with small receptive fields were always strictly ipsilateral The size of their receptive field was about 1 cm² or less and the group contained a considerable number of cells (20 %) with very precise fields having a diameter of less than 5 mm Details about the localization of the receptive fields are given in Table I

B *Latency of response to orthodromic electrical stimulation*

The shortest latency of our responses recorded at the level of N V snpr from primary afferent fibres in the trigeminal tract was 1 1 msec which corresponds to a conduction velocity of 80 m/sec as the length of the peripheral path is about 90 mm Assuming a synaptic delay of 0 6 msec leads to the conclusion that all cells having a latency of response shorter than 2 3 msec should be secondary neurones The latency to electrical stimulation in the receptive field was tested in 50 cells belonging to the group of touch cells with small receptive field Out of these cells 26 (52 %) responded with a latency of less than 2 3 msec These results suggest that the majority of the above mentioned group of cells are directly discharged by fast conducting afferent fibres with conduction velocities between 50 and 80 m/sec

C *The specificity to different types of stimulus*

Touch cells with small receptive fields showed a low threshold to mechanical stimuli and a high threshold to all other stimuli tested i e they were specific to touch Thermal stimulation of the receptive field was carried out on 30 of these cells None of them responded to cooling or warming between 20 and 45°C and 24 cells (i e 80 %) were also negative to noxious cooling and warming in the receptive field which was continued until reflex movements were observed In 6 cells however the impulse frequency was increased by cooling below +10° or warming to +50°C One of these 6 cells responded to noxious cooling and warming two only to warming and 3 only to cooling The thermal responses were confirmed by repeated tests They were not due to mechanical stimulation by the thermode and as far as could be judged by visual inspection they were independent of movements within the receptive field

Nineteen touch cells with receptive fields on the tongue were tested with taste solutions (salt bitter acid and distilled water) No response was seen in 18 cells but 1 cell showed a delayed increase in impulse discharge when the acid solution was applied

In 10 cells belonging to this group the effect of sound stimuli and light flashes were tested but none of them responded. Vibration due to tapping of the stand was routinely applied as responses to this stimulus occurred above and below the trigeminal nucleus. Touch cells with small receptive fields never responded to this stimulus.

D. *Response pattern*

The touch cells with small receptive fields generally adapted rapidly to a maintained light mechanical stimulus. In 39 (74 %) of 53 investigated cells the response was thus phasic and consisted of a short burst of spikes. In 3 of them another burst was seen when the stimulus was removed. The remaining 14 cells (26 %) were slowly adapting and showed a persistent discharge to maintained touch or slight maintained bending of hairs.

In the group of 39 rapidly adapting touch cells 34 were tested by application of a strong steady pressure. The response was nevertheless phasic in 21 of them but 13 cells developed a persistent discharge in response to this stimulus. The fast adapting as well as the slowly adapting groups were represented at all rostro-caudal levels within NVsnpr and NVspo.

Cells responding to movements of the vibrissae

The cells responding to movements of the vibrissae may be looked upon as closely related to the group of touch cells with small receptive fields described above, as they receive precisely localized tactile information from the periphery. However, considering the rather specialized function of the vibrissae it was decided to treat them as a separate group.

The nuclear topography of these cells is interesting because they are very rare in the rostral half of NVsnpr, i.e. at the level of NVmt. Fig. 8 A shows just 4 cells lying along the lateral border of NVsnpr. The significance of this small number may be appreciated if compared with the large number of cells investigated in this part of the nucleus (cf. Fig. 7 B). In the overlapping part of NVsnpr caudal to NVmt a large number of whisker cells were found. Their distribution is identical with that of the previously described touch cells, with a column of maxillary whisker cells and another of ophthalmic whisker cells both in the same positions as the maxillary perioral and periorbital touch cell columns respectively. In this same region several whisker cells were found medially to NVsnpr in NVspoγ (Fig. 8 B). In this respect also the distribution agrees with

that of the touch cells Caudal to NVsnpr the columns of whisker cells continue through the lateral part of NVspoγ just as do the columns of touch cells Their localization together with the last mentioned group of cells is shown in Fig 8 C in which cells belonging to the two groups are plotted together

In all 38 cells responding to movement of whiskers were investigated and 28 of these were discharged by maxillary vibrissae 8 by ophthalmic and 2 by vibrissae on the cheek 25 cells were localized to NVsnpr and 13 to NVspoγ The receptive field was confined to one or two vibrissae in 48 % of the whisker cells found in NVsnpr but only 8 % (1 out of 13) of the cells localized to NVspoγ had such a well defined receptive field The majority of the whisker cells responded to several vibrissae and some of them either to all ipsilateral maxillary or to all ipsilateral ophthalmic vibrissae

The latency of the response to electrical stimulation in the receptive field was investigated in 9 whisker cells Only 2 of these responded with a latency shorter than 2 3 msec Thus some of the cells in this group are obviously activated directly by fast conducting afferent fibres The observations however are too few to allow conclusions concerning the relative size of this part of the group

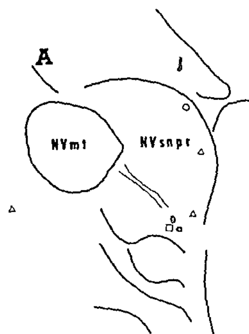
The whisker cells were occasionally tested by the application of thermal stimuli sound or light flashes but in no case was a positive response observed

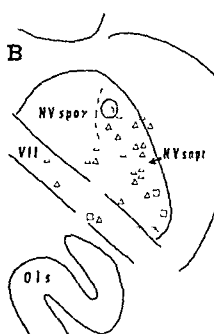
The response pattern was investigated in 19 whisker cells Great sensitivity slow adaptation and a steady discharge to maintained bending of the whisker were found in 11 cells but 8 cells were rapidly adapting and also less sensitive 50 % of the cells showed directional sensitivity

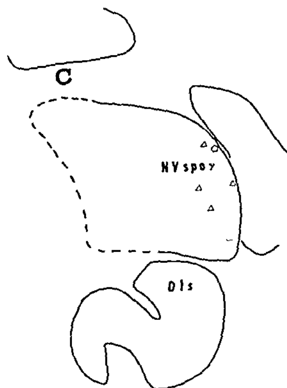
Cells with periodontal receptive fields

The central region in NVsnpr is occupied by a column of cells responding to mechanical stimulation of the teeth as shown in Fig 7 A (Λ) The stimulus was a light tap of a tooth or moderate pressure on the gingiva near the root of a tooth often effective from either side of the alveolar process These cells were generally very sensitive and some of them responded even to a stroke of a soft brush on the tooth It was concluded that the receptors were located in the periodontal membrane (PFAFFMAN 1939)

The column of periodontal cells continues caudally into NVspoγ (Fig 7 C) and was observed in this nucleus in the overlapping region as well as caudal to NVsnpr Only occasional periodontal responses were recorded from the laterally situated caudal overlapping half of NVsnpr


A
NVmt
NVsnpr


B
NVspoy
VII
NVsnpr
Ols


C
NVspoy
Ols

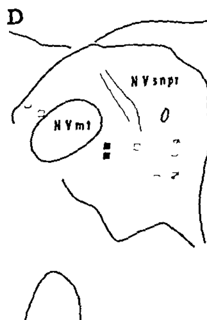

D
NVsnpr
NVmt

Table II The location of the receptive fields of cells activated by periodontal receptors

	NVsnpr	NVspoy	Total
Mn periodontal	11 cells	7 cells	18 cells
Mx	6	3	9
Mn Mx	2	5	7
Total	19 cells	15 cells	34 cells

The receptive field of the periodontal cells was always restricted to the ipsilateral side It could be rather small and was sometimes confined to one tooth However there was frequently convergence from several teeth and sometimes from all ipsilateral incisors and molars As seen in Table II convergence from mandibular and maxillary teeth was also found

The latency of the response to electrical stimulation in the receptive field was investigated in 13 cells belonging to this group Out of these 5 showed a latency shorter than 2 3 msec and all of these were found in NVsnpr The results suggest that a considerable part of the periodontal group in NVsnpr consists of cells directly discharged by rapidly conducting afferent fibres

Pressure cells with small receptive fields

Cells responding to moderate pressure were found scattered among the touch cells in NVsnpr and NVspoy They were localized to the appropriate columns of oral perioral or periorbital touch cells according to the loca

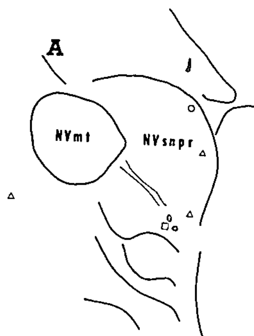
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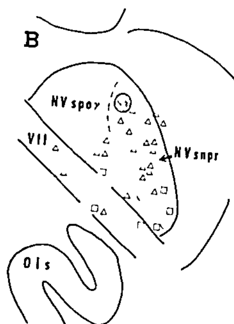
Fig 8

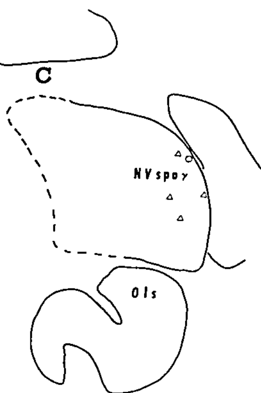
A and B Diagrams summarizing the distribution of whisker cells found in NVsnpr at the level of NVmt (A) and in the region of overlap of NVsnpr and NVspoy (B)
(○)=cells responding to movement of cheek whiskers
(△)=cells responding to movement of maxillary whiskers
(□)=cells responding to movement of ophthalmic whiskers

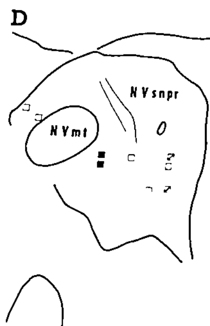
C Distribution of whisker cells and of touch cells with small receptive fields found in NVspoy caudal to NVsnpr
(○)=touch cell with mandibular receptive field
(△)=touch cell with maxillary receptive field or maxillary whisker cells
(□)=cell responding to movement of ophthalmic whiskers

D Distribution of cells with receptive fields including the cornea found in NVsnpr near the caudal pole of NVmt
(■)=touch cells with receptive field restricted to cornea
(⊡)=touch cells with receptive field restricted on cornea and eyelids
(◻)=cells with wide receptive fields responding to touch of the cornea and to noxious stimuli on other parts of the receptive field


A
NVmt
NVsnpr


B
NVspoy
VII
NVsnpr
Ols


C
NVspoy
Ols


D
NVsnpr
NVmt

was shown by four of these cells. Their receptive field was found apically on the ipsilateral side of the lower and the upper lip. The borders of the field were well defined and these cells were discharged by light touch. The latency of their response to electrical stimulation of the upper and the lower lip was the same and was shorter than 2.3 msec (1.7 and 2.0 msec) in two of them. It may therefore be assumed that they were discharged directly by fast conducting afferents from both lips. In the nucleus they were localized to the border zone between the mandibular and maxillary touch cell columns.

In all ten of the wide receptive touch cells were tested with electrical stimulation in their receptive field and only three (including the two mentioned above) responded with a latency shorter than 2.3 msec.

An exception from the rule of strictly ipsilateral connections was found in this group of cells. One of them was discharged by touching the nictitating membrane, the inside of the nostril and the lower lip on the contralateral side. It was localized to the medial part of NVspoy.

Cells responding to noxious stimuli

A considerable group of cells, 49 in all, responded to noxious stimuli of mechanical (pinching with forceps, pinprick) and sometimes also of thermal (+10°C or +50°C) type. These cells were scattered over the central and the medial part of NVsnpr (Fig. 9A and B, X). Several were found in regio H of MEESSEN and OLSZEWSKI (1949), i.e. in the cell poor zone immediately surrounding NVmt. Although some of them were found among the touch cells in the topographically organized columns, in general they were rare in this region. This was also true at the level of over lap between NVsnpr and NVspo. As shown in Fig. 9C (X), many nociceptive cells were found in NVspoy, whereas the laterally situated and topographically organized NVsnpr contained only few such cells. Fig. 9D indicates that the same organization persists also in the part of NVspo, situated caudal to NVsnpr.

According to size of the receptive field the nociceptive cells could be divided into a group with rather small fields localized to a part of one dermatome and another group with fields exceeding the borders of one dermatome. The first mentioned was the smaller of the groups and consisted of 10 cells, 8 of which had their receptive fields on the tongue. They all responded to noxious mechanical stimulation (pinch) but only from a small ipsilateral receptive field. They never responded to taste stimuli. Noxious thermal stimuli were tried on 7 cells. One of them showed an

Table III The location of the receptive fields of cells responding to localized pressure or stretch

Receptive field	Pressure	Stretch
NV *snpr*		
T	15 cells	3 cells
Bucca		2
Perioral Mn	3	2
Perioral Mx	6	
Periorbital	2	
NV *spoy*		
T	3	
Bucca		2
Perioral	2	
Total	*31 cells*	*9 cells*

tion of their receptive field which was ipsilateral and always restricted to a part of one of the trigeminal dermatomes The number of the observed pressure cells as well as the location of the receptive fields are given in Table III which also contains a small number of cells responding to stretch of the tongue bucca or lip Most of the pressure cells were discharged from the tongue

The latency of the response to electrical stimulation in the receptive field was tested in 7 cells and was shorter than 2 3 msec in 3 of them It may therefore be concluded that at least part of this group consisted of secondary neurones

The pressure cells did not respond to noxious thermal stimuli Taste stimuli were applied to those discharged from the tongue but they never responded Slow adaptation to maintained pressure was most commonly observed but a number of rapidly adapting pressure cells were also found

Touch and pressure cells with wide receptive fields

In NV *snpr* and NV *spoy* there was a small group of cells responding only to touch (22 cells) or in other cases only to moderate pressure (3 cells) *but from a wide receptive field* which exceeded the borders of one trigeminal dermatome No responses to noxious thermal or to gustatory stimuli were found in this group

The distribution of these wide receptive cells within the nuclei is shown in Fig 9 (○) They are obviously scattered all over NV snpr and NV spoy Some of them appeared in the dorsally and laterally situated columns of touch cells with small receptive fields An interesting type of convergence

was shown by four of these cells Their receptive field was found apically on the ipsilateral side of the lower and the upper lip The borders of the field were well defined and these cells were discharged by light touch The latency of their response to electrical stimulation of the upper and the lower lip was the same and was shorter than 2 3 msec (1 7 and 2 0 msec) in two of them It may therefore be assumed that they were discharged directly by fast conducting afferents from both lips In the nucleus they were localized to the border zone between the mandibular and maxillary touch cell columns

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An exception from the rule of strictly ipsilateral connections was found in this group of cells One of them was discharged by touching the nictitating membrane the inside of the nostril and the lower lip on the contralateral side It was localized to the medial part of NV spoγ

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According to size of the receptive field the nociceptive cells could be divided into a group with rather small fields localized to a part of one dermatome and another group with fields exceeding the borders of one dermatome The first mentioned was the smaller of the groups and consisted of 10 cells 8 of which had their receptive fields on the tongue They all responded to noxious mechanical stimulation (pinch) but only from a small ipsilateral receptive field They never responded to taste stimuli Noxious thermal stimuli were tried on 7 cells One of them showed an

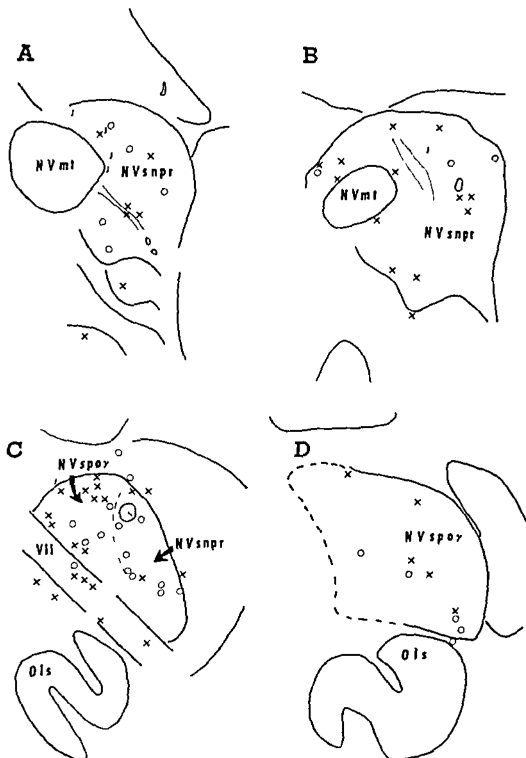


Fig 9 Diagram showing distribution of cells responding to noxious stimuli ( ) to touch within wide receptive fields (( ) or to jaw movement (J)

A Cells found in NVsnpr more than 190 μ rostral to the caudal pole of NVmt

B Cells found in NVsnpr at the caudal pole of NVmt

C Cells found in region of overlap of NVsnpr and NVspoγ

increased discharge frequency when +8°C was applied to the tongue. It did not respond to noxious heat. The other 6 cells did not respond to either noxious cooling or warming. Rapidly as well as slowly adapting responses to pinch were observed in the group.

The majority of the nociceptive cells (39 out of 49) had a wide receptive field. Although the receptive field exceeded the borders of one trigeminal dermatome it was generally ipsilateral and confined to the face. Exceptions from this rule were, however, observed in 9 cells. Eight of these cells were discharged also by pinching the paws or the pinna. A bilateral receptive field on the face was observed in 4 of them. All these 9 cells were localized to the medial or ventromedial part of the nucleus.

The B cells described by GORDON, LANDGREN and SEED (1961) had a more sensitive region within the receptive field. This was also observed in the group of wide receptive nociceptive cells. The most sensitive region responded to touch in 14 cells but moderate pressure was required in 15 cells. Ten cells responded only to noxious stimuli and apparently lacked a region of higher sensitivity.

The B cells of GORDON *et al.* often had the most sensitive part of the receptive field localized to the medial angle of the eye. In the present population of cells, obtained further rostrally in the nucleus, it was more common to find the most sensitive region on the tongue, nose or lips. Oral structures (generally tongue) were included in the receptive field in 71 % (35 of 49) of all the nociceptive cells.

Noxious thermal stimuli were applied to the receptive field of 25 nociceptive cells with wide receptive fields. No response was seen in 17 cells but 8 cells were discharged by noxious warming and 3 of them also responded to noxious cooling. Tests with taste solutions, light flashes and sound stimuli were tried in 4 cells but none of them responded.

The cells of this group always showed a slowly adapting discharge in response to noxious stimuli. The latency of the response to electrical stimulation in the receptive field was investigated in 24 nociceptive cells and only 3 of these had a latency shorter than 2.3 msec. The latency of the rest varied between 2.3 and 8.4 msec.

Cells responding to movements of the lower jaw

A small group of cells were observed some of which responded to opening and some to closing of the mouth. The receptors activating these cells could obviously have been located in greatly different structures and a

closer identification of them was not attempted It is, however of some interest to note that the cells were localized to the immediate surroundings of NVmt (regio H) See Fig 9 A symbol J

Responses from bordering nuclei

On the way down to the trigeminal nucleus the recording tracks passed through cerebellum the cerebellar nuclei and the vestibular nuclei Just ventrally to the fifth nucleus they entered into *nucleus olivaris superior* Some characteristic responses from the bordering nuclei were noted and served as an aid in the preliminary localization of the borders of the trigeminal nucleus Spontaneously active units with large spikes were thus recorded from N Bechterew and from Deiters nucleus These units never responded to our stimuli In a cat showing nystagmus it was noted that a number of such units increased their discharge frequency in phase with the movements of the eye These units were localized to N Bechterew Responses to vibration (tapping the stand or the skull) were sometimes observed in the vestibular nuclei just before entering the trigeminal nucleus Such responses were regularly recorded from *nucleus olivaris superior* They were easily recognized and appeared suddenly when the track left the trigeminal nucleus In the histological reconstructions they were nicely localized to the S shaped superior olive

Responses to touch and pressure on the face were occasionally recorded from the region of the cerebellar nuclei In one experiment a group of 6 units showing such responses were localized just ventral to the border zone between N *interpositus* and *N dentatus* Two of them responded to touch and four to pressure Their receptive fields were located on the face but they received convergence from more than one of the trigeminal dermatomes The latency of their response to electrical stimulation was long (7 5—160 msec)

Discussion

The functional division of the trigeminal nucleus proposed by GERHARD (1923) and SJÖQVIST (1938) is attractive because of its simplicity. According to their theory all relays essential to tactile perception are located in the main sensory nucleus and those essential for the reception of pain and temperature are located in the nucleus of the Spinal trigeminal tract. A functional subdivision of the long trigeminal cell column in transverse segments is thus suggested. However, the present results disagree with such an interpretation. They suggest that cell columns of similar function are arranged longitudinally. Topographically arranged longitudinal columns of cells sensitive to touch within small receptive fields thus extend from the rostral pole of the main sensory nucleus over into the rostral subdivision of the nucleus of the spinal trigeminal tract. The studies of GORDON, LANDGREN and SEED (1961), KRUGER, SIMINOFF and WITKOVSKI (1961) and WALL and TAUB (1962) show that these columns continue through the middle and the caudal part of the nucleus of the spinal trigeminal tract. The nerve impulses arriving in the trigeminal touch fibres are thus spread out over the major part of the trigeminal nucleus, a finding which is suggested by the bifurcation of the coarse trigeminal afferents described by CAJAL (1952). In fact no unique functional property is localized to the main sensory nucleus according to our observations. This is in agreement with the view of ÅSTRÖM (1953), who emphasizes that the ascending collaterals to the main sensory nucleus are just the first of many similar branches given off by the afferent fibre during its course along the spinal trigeminal tract.

When preparing this report for publication the recent work of KRUGER and MICHEL (1962 a, b and c) came to our knowledge. Our findings concerning the longitudinal and topographical organization of the touch cells within the trigeminal nucleus confirm their results. The description of the cells in the main sensory nucleus given by WALL and TAUB (1962) on the other hand disagrees to some extent with our results as well as with those of KRUGER and MICHEL. According to WALL and TAUB the cells in the main sensory nucleus have large receptive fields generally

located on the face skin and less often in the mouth and the topographical organization is less apparent here than it is in the middle part of the nucleus of the spinal trigeminal tract. The fact that anaesthetized preparations were used in our *series cannot explain* the disagreement because the experiments of KRUGER and MICHEL were made on decerebrate animals and produced results consistent with ours. The wide receptive type of cell described by WALL and TAUB was observed also by us (the nociceptive cells with wide receptive fields). In our experience these cells *are not* however evenly distributed all over the transverse section of the nucleus but are more common in its central and medial parts. The percentage of spontaneously active cells in the nucleus was around 60 in our series as well as in that *of* WALL *and* TAUB *and it therefore* seems probable that the degree of depression of the activity in the two *types of preparations is of the same order of* magnitude.

The topographically organized columns of touch cells occupy a dorsal and lateral shell of the nucleus. The majority of the cells within this shell are *secondary neurones* and are *apparently directly discharged by* first conducting afferent fibres only. The cells found in the medial part of the nucleus showed a much more complex type of convergence from large *receptive fields* probably *involving different types of* afferents. There was even evidence of a greater complexity the further medially the cell was located. Some cells discharged from most parts of the body were thus found near the border between the trigeminal and the reticular nuclei.

KRUGER and MICHEL (1962a, b) found no rostro caudal organization *within the longitudinal cell columns. In our experience there is* however certain evidence of such an organization. The small number of whisker cells in the rostral half of the main sensory nucleus compared to the large number in the caudal half is one example, the difference in the distribution of the touch cells discharged from the lips is another. *A detailed investigation of large cell populations within continous parts* of the columns will probably reveal further inequalities in the rostro caudal distribution of the different cell types. It is possible that such *inequalities* could explain the puzzling findings of DARIAN SMITH and MAYDAY (1960) which they interpreted as evidence of a double representation of the face *within the nucleus of the spinal trigeminal tract*.

It is necessary to consider the role that the described cell types may play in perceptive or reflex mechanisms in spite of the fact that any *suggestions* concerning function will be highly speculative. A cell which is discharged with a minimum of delay from a small, well circumscribed receptive field by *one type of stimulus only* has *properties* expected in a neurone subserving perceptive functions. These features are found in

a majority of the touch cells in the longitudinal cell columns. It is therefore suggested that these cells are links in the chain of neurones subserving tactile perception. There is no reason to believe that this function is restricted to the main sensory nucleus. Still more evidence pertinent to this question will be presented in a following paper in which the trigeminal projection to the thalamus will be considered (LISSMAN, FROMM, LANDGREN and NOVIN 1964).

According to WALL and TAUB (1962) and to KRUGER and MICHEL (1962 b) trigeminal cells responding only to noxious stimuli are very rare or do not exist. In the present material, however, 20 out of 49 nociceptive cells belonged to this group and they were all localized to the trigeminal nucleus. It is not possible to explain the nociceptive responses as artefacts (cf. KRUGER and MICHEL 1962 b) due to movements between the brain and the electrode caused by reflex changes in the blood pressure because noxious stimuli applied outside the ipsilateral receptive field were without effect. In some cases it was further observed that noxious heat applied to the receptive field did not discharge a cell which was fired by noxious mechanical stimulation.

Some nociceptive cells were directly discharged by fast conducting afferents from the most sensitive part of their receptive field but most of them showed longer latencies. Noxious mechanical and thermal stimuli could affect these cells either directly via slowly conducting afferents or indirectly via polysynaptic connections. On the basis of the present evidence it is impossible to decide whether such cells are secondary or higher order neurones. This conclusion was also emphasized by GORDON, LANDGREN and SEED (1961) when discussing their B cells. The nociceptive cells with wide receptive fields found in the present series to be located near the trigeminal motor nucleus are probably interneurones in local reflex arcs. Similar cells located medially near the border with the reticular nuclei may have other so far unknown functions. In our opinion there is no *a priori* reason to exclude the possibility that cells of this type could be involved in mechanisms subserving the perception of pain. Our present knowledge based on results obtained from a selected sample of neurones is, however, not sufficient to allow the presentation of theories concerning the central mechanisms of pain.

JOHANNES MÜLLER's (1835) law of the specific sensory energies was extended by M. BLIX (1882) and by MAX VON FREY (1895) to the skin sensations. The validity of this extension has, however, been much debated. As a consequence of JOHANNES MÜLLER's theory the sensation of touch should depend on the activity in receptors and afferent nerve fibres activated exclusively by light mechanical deformation of the skin. These

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*fibres should in turn be connected to chains of neurones within the cen*tral nervous system also activated by this type of stimulus only. Do such specific paths exist? In order to answer this question it is necessary to consider the evidence concerning the specificity of skin afferents.

HENSEL and ZOTTERMAN (1951 a) and DODT (1953) have shown that single afferent fibres from the cat's tongue may be activated by tactile as well as by thermal stimuli within the physiological range. This type of afferent is rather common in the saphenous and the sural nerves of the cat as demonstrated by WITT and HENSEL (1959) and by HUNT and MCINTYRE (1960). Such findings made it necessary to reexamine the mechanoceptors paying particular attention to their sensitivity to thermal stimuli. This was done in a number of recent papers (BOMAN 1959, HENSEL and BOMAN 1960, IGGO 1960, LOEWENSTEIN 1961) and it has been shown that a large number of sensitive mechanoceptors are unaffected by thermal stimuli or in other cases show a high threshold *to such stimuli. The experiments of BOMAN (1959) are of particular* interest for the present discussion because they were carried out on sensitive mechanoceptive fibres in *n. infraorbitalis* of the cat. These fibres were unaffected by rapid warming and cooling between 20° and 50°C.

Afferent fibres responding to cooling or to warming of the receptive surface but not to mechanical stimuli were described by ZOTTERMAN (1936), HENSEL and ZOTTERMAN (1951 b), DODT and ZOTTERMAN (1952), MARUHASHI, MIZUGUCHI and TASAKI (1952), HENSEL, IGGO and WITT (1960) and by IRIUCHIJIMA and ZOTTERMAN (1960). The existence of large groups of skin afferents specific to mechanical or to thermal stimuli is thus proven *beyond reasonable doubt*.

The specificity of the central neurones in the lemniscal system was emphasized by MOUNTCASTLE (1957, 1961) who based his opinion on experiments with thalamic and cortical neurones tested with different types of mechanical stimuli. Thermal stimuli were not applied in his experiments. *This fact may possibly explain why they have failed to con*vince MELZACK and WALL *(1962) who state "there is no evidence that* would allow us even at central levels to assume that each pathway carries information about a single physical dimension".

In the present series a particular point was made of applying mechanical and thermal as well as other types of stimulus. The results clearly demonstrate the existence of a large group of secondary neurones in the rostral part of the trigeminal nucleus activated only by light mechanical stimulation of their receptive field. *These neurones are specific to the* stimulus of touch in the sense that the threshold of their activation by *this stimulus* was low, whereas the threshold of all other stimuli used

were high Their threshold to thermal stimuli was obviously outside the physiological range as cooling below +10°C and warming to 50°C which gave rise to reflexes of nociceptive type did not discharge the cells These findings are in agreement with the observations of GORDON LANDGREN and SEED (1961) made on a similar group of cells (their A cells) in the caudal part of the nucleus of the spinal trigeminal tract Only a small group of cells responding to mechanical and to thermal stimuli in a way similar to that seen in certain afferent fibres was found in the present experiments The specific cells were however much more common and formed about 80 % of the group referred to above as "touch cells with small receptive fields"

Thalamic neurones discharged by the ascending trigeminal paths were investigated by LANDGREN (1960 a and b) and neurones in the cortical projection area of the tongue have also been examined (LANDGREN 1957 a and b) At the thalamic and cortical levels of the trigeminal path the majority of the cells were specific to mechanical stimuli and a smaller but still considerable group of cells responded only to cooling of the receptive field on the tongue (cf LANDGREN 1961 p 440 table 1) Specific thermoceptive cells have not so far been found within the trigeminal nucleus a fact that serves mainly to emphasize that our sample of cells is not entirely representative of the trigeminal population Nevertheless the above described series of investigations allow the important conclusion that chains of neurones specific to tactile or to thermal stimuli reach the cortical level within the trigeminal system of the cat and this conclusion is in full agreement with the principle of JOHANNES MÜLLER

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From the Swedish Medical Research Council Unit for Pediatric Hematology the Department of Pediatrics the Department of Medicine and the Department of Physics University of Uppsala Sweden

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Introduction

The introduction of suitable radioactive isotopes of iron has resulted in a great number of investigations concerning the kinetics of distribution of radioiron within the body in normal and pathological conditions. The aim of such studies has been to characterize certain aspects of iron metabolism and to get a further insight into the kinetics of the red cell mass, i.e. the production and destruction of red blood cells.

Most of the published work has been of a descriptive nature and very few attempts have been made to interprete the tracer data in terms of the simple and fundamental physiological properties of the system, i.e. the sizes of metabolic iron pools and the flows of iron between the pools. One notable exception is the work of POLLYCOVE and MORTIMER (1961); this will be discussed below. Since it is reasonable to assume that the degree of complexity of the real system is quite large, i.e. the number of distinct pools of iron is probably quite large, any attempt to describe the system in terms of a quantitative model must necessarily be looked upon as an oversimplification. However, it should be remembered that the function of a model is only to fix the current ideas into a rigorous framework and to provide a basis for new experiments; these will inevitably prove the inadequacy of the model.

The present paper describes the mode of analysis and the resulting properties of the simplest model that can be constructed on the basis of the available data.

Theoretical part

1 *The structure of the model*

The model which will be considered here is shown in figure 1. This model, although simple, seems to contain all the elements necessary to satisfy the experimental data so far collected. In particular, as will be shown in the present work, it will satisfy all the available tracer data. It is, of course, trivial to note that a more complicated model, with the same basic structure, will also fulfill these requirements.

The present model is similar to that adopted by POLLYCOVE and MORTIMER (1961), but differs from the latter in one important aspect: no *a priori* assumption is made here concerning the pathway of the return of iron derived from senescent red cells. POLLYCOVE and MORTIMER assume, although it is not explicitly stated, that this iron is directly returned to the plasma iron pool. As will be shown in the following, the question of the pathway of this flow of iron is of decisive importance for the calculation of the flows in the system.

2 *The stationary state*

Restricting our analysis to the stationary state, i.e. the pool sizes and the flows are constant with respect to the time, we have (for interpretation of the designations see fig. 1)

$$J_{SC}+J_{SP}=J_{PS} \qquad (1\ a)$$

$$J_{PS}+J_{PC}+J_{PM}=J_{SP}+J_{MP} \qquad (1\ b)$$

$$J_{MP}+J_{MC}=J_{PM}+J_{PrM} \qquad (1\ c)$$

$$J_{PrC}+J_{PrM}=J_{S} \qquad (1\ d)$$

$$J_{SC}+J_{PC}+J_{MC}+J_{PrC}=J \qquad (1\ e)$$

We furthermore define flow rate constants k such that

$$k_{SP}=J_{SP}/P \qquad (1\ a)$$

$$k_{PS}=J_{PS}/S \qquad (2\ b)$$

$$k_{PM}=J_{PM}/M \qquad (2\ c)$$

$$\lambda_{MP} = J_{MP}/P \qquad (2\ d)$$

$$\lambda_{PrM} = J_{PrM}/M \qquad (2\ e)$$

where S P and M denote the pool sizes

3 General mode of analysis

If the proposed model is in fact the correct model the dynamic properties of it i e the numerical values of the flow rate constants λ can be calculated from the behaviour of the labelled iron in the plasma pool if and only if it is assumed that the pools are rapidly mixed (cp SHEPPARD 1962) This assumption is by no means trivial (see for instance BERGNER 1962) but has to be made in order to extract meaningful information from the tracer data Since there is obviously some uncertainty both concerning the structure of the model and in particular

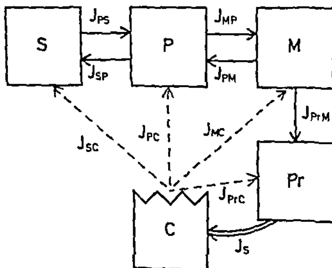


Figure 1 The structure of the model for ferro erythrokinetics S = storage iron pool (liver spleen etc) P = plasma iron pool M = bone marrow iron pool i e that part of the bone marrow iron that is in reversible exchange with the plasma iron Pr = red cell precursor heme iron pool C = circulating red cell iron pool The flows J_{SC} J_{PC} J_{MC} and J_{PrC} denote the iron flow from senescent red cells Obviously $J_{SC} + J_{PC} + J_{MC} + J_{PrC} = J_S$ is the total effective hemo lobin synthesis iron flow Asterisks as superscript to the letters denote the amount and th flow of radioactive iron.

concerning the mixing properties of the pools the use of plasma radioactivity data alone does not seem to be realistic In the present approach, plasma tracer data *and* data on the appearance of the tracer in peripheral blood cells i e pool C have been used In this way it would appear that certain properties of the system can be revealed also when some freedom is given with respect to the mixing properties of the various pools This approach differs from that of POLLYCOVE and MORTIMER (1961) who used plasma data only The use of data on the appearance of the tracer in peripheral red cells makes it necessary to develop a theory for the uptake of iron in the red cell precursors and the time course of the release of such cells from the marrow This part of the analysis is given below (section 5)

4 The plasma radioactivity disappearance curve

The model depicted in figure 1, together with the assumption of pool homogeneity constitutes a three compartment open system that can be solved with conventional methods (see for instance SKINNER CLARK BAKER and SHIPLEY 1959 SHEPPARD 1962) It should be noted that the time lag for the return of tracer by way of the flows J_{SC} J_{PC} J_{MC} and J_{PrC} is so large that for all practical purposes the system can be treated as being open in the present context although it is actually closed The solution for the plasma radioactivity P^* is

$$P^*_t = P^*_0 \sum_{i=1}^{n} H_i \, e^{-g_i t} \qquad (n = 3) \tag{3}$$

where

$$\sum_{i=1}^{n} H_i = 1 \tag{4}$$

and H_i and g_i are constants

The flow rate constants λ are contained in the constants H_i and g_i in such a way that the latter uniquely determine the values of the former

It is an experimental fact (POLLYCOVE and MORTIMER 1961) that the plasma radioactivity disappearance curve in normal individuals can be well described by an expression such as (3) and (4) where $H_1 > H_2 > H_3 > 0$ and $g_1 > g_2 > g_3 > 0$ Limitations of the amount of radioactivity that can be given to normal individuals have so far prevented an analysis of such an accuracy that the form of (3), and thus of the

model can be subjected to a critical test In fact both the precision and the accuracy of the observed plasma radioactivity in that part of the curve where H_2 H_3 g and g_3 are "dominating" is rather low

5 *The red cell radioactivity appearance curve*

The transfer of the tracer from the red cell precursor pool Pr to the circulating red cell pool C cannot be given by any ordinary first order reaction equation since there is a finite transit time for the precursors in the marrow Instead the following mode of analysis seems more realistic and yet simple enough to be handled numerically

The red cell precursor pool Pr, defined in the present model as *non* exchangeable heme iron takes up iron irreversibly from the pool M (or the pool P if the size of the pool $M = O$) The latter pool is defined here as exchangeable iron in the marrow its morphological localisation may or may not be in the precursors

The red cell precursors take up iron irreversibly from the pool M during a time period of T days At the end of T they are delivered into the circulation There may well be non iron uptaking precursors existing for a period of λ days preceding the period *of iron* uptake so that the total time spent in the bone marrow by a red cell precursor is $\lambda + T$ days The length of the time period T is not well known but it seems most unlikely that it is less than 3 days and more than 5 days This estimate and the data underlying its proposition have been discuss ed by REIZENSTEIN (1962) The rate of uptake *of iron per unit* of pre *cursor* stage during the time interval T is assumed to be uniform The approximative nature of this assumption has been discussed by LAJTHA and OLIVER (1960) Moderate deviations from this *simplified* assump tion will not materially influence the conclusions *reached in* this connection

Let us consider a set of precursors that will reach the point of delivery *to* the pool C during the time *interval* du at the time t u after the pool M has been labelled $(t = O)$ During the time interval $t = O$ the set of precursors will *take* up the same amount of iron at any one time *Accordingly* they will take up a constant fraction du of the *labelled* iron atoms which are flowing from pool M to pool Pr during the time interval dt *Thus* the number of labelled iron atoms which are taken *up* by the set of precursors under consideration is given by

$$\frac{du}{T} J^*_{PrM} \; dt \tag{5}$$

where J^*_{PrM} is the flow of radioactive iron atoms from M to Pr

The total amount of radioactivity taken up during the time interval between $t = O$ and $t = u$ then becomes

$$\frac{du}{T} \int_0^u J^*_{PrM} \; dt = \frac{du}{T} \int_0^u \lambda_{PrM} \; M^* \; dt \tag{6}$$

where M^* is the number of radioactive atoms in M

All sets of precursors that are delivered to the pool C during the time $t = O$ to $t = v < T$ will thus carry the following amount of labelled atoms

$$\frac{\lambda_{PrM}}{T} \int_0^v \int_0^u M^* \; dt \; du \tag{7}$$

This expression is valid for the case that the precursors under consideration were born before $t = O$ i e $u < T$

If the cells were "born" at the time $u - T > O$ they have been able to take up labelled iron atoms during the whole time of their maturation They will therefore deliver to the pool C

$$\frac{\lambda_{PrM} \; du}{T} \int_{u-T}^u M^* \; dt \tag{8}$$

labelled atoms

The cells that are delivered during the time T to $v > T$ will thus bring to the pool C

$$\frac{\lambda_{PrM}}{T} \int_T^v \int_{u-T}^u M^* \; dt \; du \tag{9}$$

labelled atoms

The cells that are delivered from $t = O$ to $t = v > T$ will thus bring to the pool C a number of labelled atoms equal to the sum of (7) and (9)

The amount of tracer in M at any one time is given by

$$M^*_t = M^*_0 \sum_{i=1}^{n} K_i \, e^{-g t} \qquad (n = 3) \tag{10}$$

where

$$\sum_{i=1} K_i = 0 \tag{11}$$

and the expressions (7) and (9) can therefore be integrated The result is

1 For $t = 0$ to $t = v < T$

$$\frac{C^*_t}{P^*_0} = \frac{k_{PrM}}{T} \sum_{i=1}^{n} \frac{K}{g} \left(v + e^{-gv} - 1\right) \tag{12}$$

2 For $t = T$ to $t = v > T$

$$\frac{C^*_t}{P^*_0} = \frac{k_{PrM}}{T} \sum \frac{K_i}{gg} \left(g T - e^{-g(v-T)} + e^{-gv}\right) \tag{13}$$

Thus the amount of tracer in the circulating red cells C^*_t at any one time is given in terms of the constants g K and T (the constant k_{PrM} is contained in g_i and K_i) It is thus possible in principle to evaluate the values of these constants from the experimentally observed quantities C^* and $v = t$

6 *Computations*

General The data obtained in the present study on the appearance of radioiron in circulating red cells in normal individuals (see experimental part) were used in the following manner Within the normal range of radioiron uptake two typical curves were constructed as seen in figure 2 From each of the two curves 14 paired values of the fraction of injected dose present in the circulation on each day were fed into an electronic digital computer (see below) together with the equations (12) and (13) and preselected values of T i e 3 4 and 5 days respectively Also preselected values of g_1 were given since this parameter is well known in the normal individual The following values of g_1 were chosen on the basis of the experimental data obtained in this work (see experimental part table 1) 7 0 9 0 11 0 13 0 and 15 0 day^{-1} By a curve fitting procedure described below in more detail the values of

the remaining parameters, i.e. g_2, g_3, H_1 and H_2 were determined. The values of H_3, k_1, k_2 and k_3 are then automatically given.

This procedure gives directly the values of all the flow rate constants f. The values of the flows and the pool sizes are then fixed and given for any given value of the pool size P and for any given choice of the pathway of return of iron from senescent red cells.

Specific. As mentioned above, the parameters g_i and H_i are determined by fitting the theoretical curves—predicted by the model—with the two experimental curves, i.e. the disappearance curve and the appearance curve. For this purpose the method of least squares was applied. The fact that the curves predicted by the model are sums of exponential functions implies, however, that the fitting problem becomes non-linear. Iterative procedures have to be applied, which increases the required computer time. Furthermore, the sum of the squares of the differences between the theoretical and experimental curves at the measured points is not a quadratic function. Thus there exist several minima to which the iterative methods applied may converge. Rather drastic changes in the estimated start values for the parameters g_i and H have therefore to be made in order to find the absolute minimum.

In the present case the method of least squares is, of course, somewhat academic. The series of parameter values of g and H_i which produces the "best fit" according to this criterion may not necessarily be the best from the point of view of the biologist. For this reason, and since preliminary experiments showed it important and necessary that the experimentalist can continuously follow the fitting procedure, we rejected the standard method of obtaining the mathematically best fit. Instead we developed the following procedure especially suited for a computer system as e.g. the IBM 1620. From the experiments a set of reasonable values for the parameters g_i and H_i is fed in. Using the least square method, the most critical parameter, specific for the case under investigation and usually easily found by experience, is adjusted by the computer. Thereafter the rest of the parameters are adjusted in a sequence that can be determined by the operator. This is done automatically two or three times by the computer, after which the whole procedure can be repeated with a new set of values of the parameters chosen by the operator-experimentalist on the basis of the results obtained. In this way several sets of values for the parameters are determined, all giving a relatively good fit to one experimental appearance curve. These sets are then examined with respect to their goodness of fit to the disappearance curves. Those sets which are com-

patible with both curves are then used to calculate the flows and the pool sizes according to the model As seen from the Results the sets of parameter values obtained give satisfactorily unique solutions

Preliminary investigations concerning the choice of the method were performed on a FACIT electronic computer system The final method was programmed in FORTRAN and run on IBM 1620

Experimental part

Plasma radioactivity disappearance The initial part of the disappearance of radioiron from plasma was measured in 25 healthy individuals 18 males and 7 females Between 5–10 μC of Fe^{59} as ferric citrate in physiological saline were injected into a cubital vein and samples drawn at 10 40 100 160 220 and 340 minutes after the injection Plasma was separated by centrifugation and the radioactivity measured on 4 0 ml plasma samples in a conventional well type scintillation detector Counting was performed so that the random (counting) error of the sample counts was less than 3 %

Plasma iron determinations were performed on the samples drawn at 40 and 340 minutes by using the method of HEILMEYER and PLÖTNER (1937) as modified by VAHLQUIST (1941)

Plasma volume determinations were performed by extrapolating the radioactivity values to zero time and by dividing the number of injected counts by the zero time radioactivity

Red cell radioactivity appearance The uptake pattern of radioiron in circulating red cells after an intravenous injection was determined in 13 healthy male subjects They received about 5 μC of Fe^{59} as ferric citrate in physiological saline Blood samples were drawn repeatedly for the next 14 days and the radioactivity in the red cells measured The fraction of the injected dose present at any time in the circulating blood cells was obtained from the number of counts injected and the radioactivity in the red cells Plasma volumes were estimated as above and the total red cell mass calculated from the venous hematocrit corrected by a factor of 0 91 to allow for the difference between venous and body hematocrit No correction for trapped plasma was made since the hematocrit used (International Micro Capillary Centrifuge Model MB) has been shown (GARBY and VUILLE 1961) to give less than 1 3 % of trapped plasma

Results

Plasma radioactivity disappearance data

Most of the data concerning the disappearance of the radio activity from plasma is shown in table 1

The remarkably small error of the individual rate constants calculated as the error of the slope from a least square analysis of the data indicate that there is no significant departure from a single exponential course of the disappearance curve for the first 340 minutes after injection A further test of non departure from a single exponential course was made as follows The seven observed values of the radio activity in the plasma in each subject permitted the calculation of a value of the slope in each of the 6 periods of the disappearance curve The mean value of the first three periods in each case was compared to the mean value of the last three periods in the same case The ratio of the two mean values was 0 969 with a standard error of 0 045

The reciprocal value of the slope and the mean plasma iron concentration in each experiment is shown in figure 3 The two regression lines have the equations $y = 0\ 896\ \ x + 56\ 4$ and $y = 1\ 443\ \ x + 4\ 9$ respectively

Table 1

	Mean value	*s e*
Hematocrit	42 5	0 74
Plasma volume *PV* ml	290[illegible]	
Red cell volume *RBC* ml	1874	
Initial disappearance rate constant r day⁻¹	11 1	0 71
Error of individual rate constant %	3 0	1 2
Average plasma iron concentration during experiment *Fe*, µg/100 ml	94	6 5
Plasma iron concentration at 40 minutes after injection µg/100 ml	*100*	*8 4*
Plasma iron concentration at 340 minutes after injection µg/100 ml	81	6 4
Individual difference in plasma iron concentration at 40 and 340 minutes respectively	20	[illegible] 2
Coefficient of correlation between 1/*r* and *Fe*	0 7[illegible]7	0 085
Coefficient of correlation between 1/*r* and *PV* *Fe*	0 512	0 141
Coefficient of correlation between *r* *PV* *Fe*, and *RBC*	0 783	0 077

Red cell radioactivity appearance data

The results are seen in figure 2 Also shown in the figure are the two typical curves selected for the curve fitting procedure

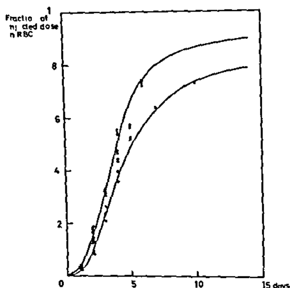


Figure ? The red cell radioiron appearance data 76 determinations in 13 in dividuals Also shown are the two curves selected for use in the fitting procedure Lower curve = curve 1 and upper curve = curve ?

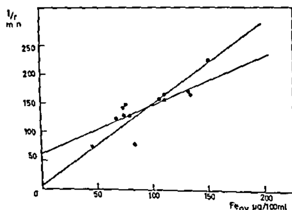


Figure 3 The relation between the reciprocal value of the slope r and the average plasma iron concentration The two re ression lines are also shown

Parameters of the model obtained from the curve fitting

The values of the constants g g_3 H_1 H_2 and H_3 obtained from the fitting procedure described previously are shown in tables 2 a and 2 b The resulting values of the flow rate constants λ are shown in tables

3 a and 3 b Also shown in tables 3 are the sums of the squared deviations from the experimental curves i e the 14 points taken from the typical curves The values of the sums show that there is a very good fit For example if the sum of the squared deviations is 14×10^{-4}, each squared deviation is about 10^{-4} and the mean deviation is about 0 01 the unit being the fraction of injected radioactivity in the circulating red cells

Table 2 a

Values of the constants g_2 g_3 H_1 H_2 and H_3 from curve 1

T	g_1	g_2	g_3	H_1	H_2	H_3
	7 0	0 282	0 00360	0 978	0 0222	0 000180
	9 0	0 279	0 00507	0 977	0 0224	0 000220
3 0	11 0	0 276	0 00762	0 977	0 0225	0 000303
	13 0	0 277	0 00923	0 977	0 0227	0 000348
	15 0	0 277	0 00975	0 977	0 0227	0 000347
	7 0	0 536	0 0635	0 972	0 0206	0 00702
	9 0	0 527	0 0641	0 973	0 0207	0 00596
4 0	11 0	0 512	0 0625	0 975	0 0201	0 00494
	13 0	0 480	0 0561	0 976	0 0204	0 00374
	15 0	0 479	0 0573	0 976	0 0204	0 00353
	7 0	0 917	0 0698	0 972	0 0200	0 00805
	9 0	0 920	0 0726	0 973	0 0200	0 00703
5 0	11 0	0 941	0 0761	0 973	0 0200	0 00655
	13 0	0 950	0 0779	0 974	0 0200	0 00602
	15 0	0 969	0 0803	0 974	0 0200	0 00574

Table 2 b

Values of the constants g_2 g_3 H_1 H_2 and H_3 from curve 2

T	g_1	g_2	g_3	H_1	H_2	H_3
	7 0	0 390	0 00410	0 986	0 0140	0 0000756
	9 0	0 386	0 00494	0 986	0 0140	0 0000755
3 0	11 0	0 382	0 00594	0 986	0 0139	0 0000781
	13 0	0 377	0 00699	0 986	0 0139	0 0000812
	15 0	0 376	0 00748	0 986	0 0140	0 0000798
	7 0	0 576	0 0266	0 979	0 0203	0 000 69
	9 0	0 565	0 0280	0 975	0 0243	0 000703
4 0	11 0	0 559	0 0296	0 981	0 0187	0 000606
	13 0	0 552	0 0304	0 981	0 0186	0 000553
	15 0	0 549	0 0306	0 981	0 0187	0 000511
	7 0	1 06	0 0603	0 980	0 0177	0 00237
	9 0	1 09	0 0683	0 980	0 0177	0 00232
5 0	11 0	1 10	0 0769	0 980	0 0177	0 00237
	13 0	1 12	0 0812	0 980	0 0177	0 00240
	15 0	1 15	0 0901	0 980	0 0176	0 00240

Table 3 a

k values from curve 1

T	g_1	k_{PS}	k_{SP}	k_{PM}	k_{MP}	k_{PrM}	Σ_{10}
	7.0	0.00439	0.833	0.167	6.00	0.261	1.3
	9.0	0.00621	0.967	0.216	7.83	0.263	2.2
3.0	11.0	0.00936	1.07	0.264	9.67	0.254	3.1
	13.0	0.0113	1.17	0.315	11.5	0.252	3.9
	15.0	0.0121	1.27	0.360	13.4	0.252	4.5
	7.0	0.100	1.90	0.195	4.90	0.473	7.8
	9.0	0.101	2.27	0.253	6.50	0.463	7.3
4.0	11.0	0.099	2.57	0.300	8.17	0.439	7.0
	13.0	0.086	2.60	0.3[illegible]0	10.1	0.408	6.8
	15.0	0.088	2.83	0.402	13.6	0.402	6.7
	7.0	0.119	2.43	0.183	4.40	0.865	21
	9.0	0.125	3.07	0.248	5.73	0.841	20
5.0	11.0	0.133	3.67	0.316	7.03	0.836	20
	13.0	0.138	4.23	0.383	8.43	0.828	20
	15.0	0.145	4.83	0.444	9.80	0.823	20

Table 3 b

k values from curve 2

T	g	k_{PS}	k_{SP}	k_{PM}	k_{MP}	k_{PrM}	Σ_{10}
	7.0	0.00455	0.533	0.0925	6.37	0.385	14
	9.0	0.00535	0.633	0.124	8.23	0.380	17
3.0	11.0	0.00649	0.733	0.154	10.1	0.377	19
	13.0	0.00757	0.800	0.183	12.0	0.371	20
	15.0	0.00828	0.900	0.213	13.9	0.367	22
	7.0	0.0316	0.800	0.136	6.07	0.570	5.7
	9.0	0.0325	0.900	0.213	7.87	0.548	4.3
4.0	11.0	0.0347	1.13	0.211	9.67	0.553	3.5
	13.0	0.0355	1.27	0.250	11.5	0.534	3.2
	15.0	0.0353	1.37	0.288	13.3	0.529	3.0
	7.0	0.0753	1.24	0.112	5.10	1.06	21
	9.0	0.0860	1.63	0.150	7.20	1.04	18
5.0	11.0	0.100	2.07	0.195	8.73	1.05	16
	13.0	0.109	2.47	0.248	10.3	1.09	16
	15.0	0.119	2.90	0.300	11.8	1.12	16

Flows and pool sizes

With the flow rate constants thus given the model is completely characterized for any given choice concerning the pathway of iron

return by way of the flows J_{SC} J_{PC} J_{MC} and J_{PC} since only one pool size needs to be known *a priori* i e the experimentally observed plasma pool size P Since we shall here be concerned with the average normal man the plasma volume will be fixed to 3000 ml and the red cell mass accordingly to 2000 ml The plasma iron pool size will then be 3 0 mg in the individual who shows an initial plasma disappearance rate constant r of 11 0 day^{-1} Since there is a strong covariation between the constant r and the plasma iron concentration (figure 3 table 1) the normal man having a low plasma iron concentration is much more likely to have a more rapid disappearance than the normal man who has a high plasma iron concentration Therefore on the basis of the data given in figure 3 we have chosen the following paired values of r (day^{-1}) and Fe (μg/100 ml) to be representative for the normal man 15 0 and 75 13 0 and 90 11 0 and 100 9 0 and 125 7 0 and 160

Tables 4–5 give the results of the pool sizes and the flows compatible with the experimental curves as well as the data of r (g_1) and the plasma pool data for given combination of the values the flows J_{SC}, J_{PC} J_{MC} and J_{PrC}

Table 4 a

Flows and pool sizes from curve 1 when $J_{PC} = J$

T	g_1	P	S	J_{PS}	J_{SP}	J_{MP}	J_{PM}	M	J_S
	7 0	4 80	911	4 00	4 00	28 8	11 2	67.3	17 6
	9 0	3 75	585	3 63	3 63	29 4	13 3	61 4	16 1
3 0	11 0	3 00	343	3 21	3 21	29 0	14 8	56 0	14 2
	13 0	2 70	280	3 16	3 16	31 1	17 3	51 0	13 8
	15 0	2 25	236	2 89	2 89	30 2	17 9	48 9	12 3
	7 0	4 80	91	9 12	9 12	23 5	6 9	35 2	16 6
	9 0	3 75	84	8 51	8 51	24 4	8 8	33 8	15 6
4 0	11 0	3 00	78	7 71	7 71	24 5	9 9	33 2	14 6
	13 0	2 70	82	7 02	7 02	27 3	12 6	36 0	14 7
	15 0	2 25	72	6 37	6 37	30 6	15.3	38 1	15.3
	7 0	4 80	98	11 7	11 7	21 1	3 7	20 1	17 4
	9 0	3 75	92	11 5	11 5	21 5	4 9	19 7	16 6
5 0	11 0	3 00	83	11 0	11 0	21 1	5 8	18 3	15.3
	13 0	2 70	83	11 4	11 4	22 8	5 8	20 5	17 0
	15 0	2 25	75	10 9	10 9	22 1	7 8	17 4	14.3

Table 4 b

Flows and pool sizes from curve 1 when $J_{SC} = J$

T	g	P	S	J_{PS}	J_{SP}	J_{MP}	J_{PM}	M	J_S
	7.0	4.80	4920	21.6	4.00	28.8	11.2	67.3	17.6
	9.0	3.75	[illegible]	19.7	3.63	29.4	13.3	61.4	1[illegible].1
3.0	11.0	3.00	18[illegible]0	17.5	3.21	29.0	14.8	56.0	14.2
	13.0	2.70	1504	17.0	3.16	31.1	17.3	54.9	13.8
	15.0	2.25	1174	15.2	2.89	30.2	17.9	48.9	12.3
	7.0	4.80	257	25.7	9.12	23.5	6.9	35.2	16.6
	9.0	3.75	239	24.1	8.51	24.4	8.8	33.8	15.6
4.0	11.0	3.00	225	22.3	7.71	24.5	9.9	33.2	14.6
	13.0	2.70	[illegible]	21.9	7.02	27.3	12.6	36.0	14.7
	15.0	[illegible]	247	21.7	6.37	30.6	15.3	38.1	15.3
	7.0	4.80	245	23.1	11.7	21.1	3.7	20.1	17.4
	9.0	3.75	225	28.1	11.5	21.5	4.9	19.7	16.6
5.0	11.0	3.00	198	26.3	11.0	21.1	5.8	18.3	15.3
	13.0	2.70	206	28.4	11.4	[illegible]	5.8	20.5	17.0
	15.0	2.25	1[illegible]4	25.2	10.[illegible]	[illegible]	7.8	17.4	14.3

Table 4 c

Flows and pool sizes from curve 2 when $J_{PC} = J$

T	g	P	S	J_{PS}	J_{SP}	J_{MP}	J_{PM}	M	J_S
	7.0	4.80	563	2.56	2.[illegible]	30.6	5.9	64.1	24.7
	9.0	3.75	442	2.37	2.37	30.9	7.6	61.3	23.3
3.0	11.0	3.00	339	2.20	2.20	30.3	8.8	57.1	21.5
	13.0	2.70	285	2.16	2.16	32.4	10.7	58.5	21.7
	15.0	2.25	245	2.03	2.03	31.3	11.5	54.0	19.8
	7.0	4.80	122	3.84	3.84	29.1	5.6	41.2	23.5
	9.0	3.75	104	3.38	3.38	29.5	8.2	38.8	21.3
4.0	11.0	3.00	98	3.39	3.39	29.0	8.0	38.0	21.0
	13.0	2.70	97	3.43	3.43	31.1	9.9	39.7	21.2
	15.0	2.25	87	3.08	3.08	29.9	10.5	36.6	19.4
	7.0	4.80	80	6.00	6.00	24.5	2.3	20.9	22.2
	9.0	3.75	91	7.82	7.82	27.0	3.4	22.7	23.6
5.0	11.0	3.00	78	7.76	7.76	26.2	4.1	21.0	22.1
	13.0	2.70	68	7.41	7.41	27.8	5.1	20.8	22.7
	15.0	2.25	55	6.53	6.53	26.6	5.7	18.7	20.9

return by w iy of the flows J_{SC} J_{IC} J_{MC} and J_{PrC} since only one pool size needs to be known *a priori* i e the experiment illy observed plasma pool size P Since we shall here be concerned with the average normal m in the pl isma volume will be fixed to 3000 ml and the red cell mass accordingly to 2000 ml The pl isma iron pool size will then be 3 0 mg in the individu il who shows an initial plasma disappearance rate const int r of 11 0 d iy^{-1} Since there is i strong covariation between the const int r and the pl isma iron concentration (figure 3 table 1) the norm il man having a low plasma iron concentration is much more likely to have a more rapid disappe ir ince th in the normal man who h is i high plasm i iron concentr ition Therefore on the basis of the d it i given in figure 3 we h ive chosen the following paired v ilues of r (day^{-1}) and I_c (μg/100 ml) to be representative for the normal man 15 0 and 75 13 0 and 90 11 0 and 100 9 0 and 125 7 0 and 160

T ibles 4–5 give the results of the pool sizes and the flows compatible with the experiment il curves as well as the dat i of r (g_1) and the pl isma pool data for given combination of the v ilues the flows J_{SC}, J_{PC} J_{MC} and J_{PC}

Table 5 a

Flows and pool sizes from curve 1 when $J_{PC} = J$

T	g_1	P	S	J_{PS}	J_{SP}	J_{MP}	J_{PM}	M	J_S
	7 0	4 80	911	4 00	4 00	28 8	11 2	67 3	17 6
	9 0	3 75	585	3 63	3 63	29 4	13 3	61 4	16 1
3 0	11 0	3 00	343	3 21	3 21	29 0	14 8	56 0	14 2
	13 0	2 70	280	3 16	3 16	31 1	17 3	54 9	13 8
	15 0	2 25	236	2 89	2 89	30 2	17 9	48 9	12 3
	7 0	4 80	91	9 12	9 12	23 5	6 9	3, ?	16 6
	9 0	3 75	84	8 51	8 51	24 4	8 8	33 8	15 6
4 0	11 0	3 00	78	7 71	7 71	24 5	9 9	33 2	11 6
	13 0	2 70	82	7 02	7 02	27 3	12 6	36 0	14 7
	15 0	2 25	72	6 37	6.37	30 6	15.3	38 1	15.3
	7 0	4 80	98	11 7	11 7	21 1	3 7	20 1	17 4
	9 0	3 75	92	11 5	11 5	21 5	4 9	19 7	16 6
5 0	11 0	3 00	83	11 0	11 0	21 1	5 8	18 3	15.3
	13 0	2 70	83	11 4	11 4	22 8	5 8	20 5	17 0
	15 0	2 25	75	10 9	10 9	2? 1	7 8	17 4	14.3

Table 5 b

Flows and pool sizes from curve 2 when $J_{MC} = J$

T	g	P	S	J_{PS}	J_{SP}	J_{MP}	J_{PM}	M	J_S
	7 0	4 80	563	2 56	2.56	30 6	30 6	331	127
	9 0	3 75	412	2.37	2.37	30 9	30 9	249	94 6
3 0	11 0	3 00	339	2.20	2 20	30.3	30.3	197	74.3
	13 0	2 60	285	2 16	2 16	32 4	32 4	177	65 7
	15 0	2 25	245	2 03	2 03	31.3	31.3	147	53 9
	7 0	4 80	122	3 84	3 84	29 1	29 1	214	122
	9 0	3 75	104	3.38	3.39	29.5	29 5	138	75 6
4 0	11 0	3 00	98	3.30	3.30	29 0	29 0	137	75.8
	13 0	2 60	97	3 43	3 43	31 1	31 1	124	66 2
	15 0	2.25	87	3 08	3 08	29 9	29 9	104	55 0
	7 0	4 80	80	6 00	6 00	24 5	24.5	219	232
	9 0	3 75	91	7.82	7 82	27 0	27 0	180	187
5 0	11 0	3 00	78	7 76	7 76	26 2	26.2	134	141
	13 0	2 60	68	7 41	7 41	27 8	27 8	112	122
	15 0	2 25	65	6 53	6 53	26 6	26 6	85	99.3

Discussion

1 Validity of the model

In the present work a quantitative ferro erythrokinetic model has been proposed The basic requirements of any such model are that it must be consistent with all the available experimental data and that it does not contain any complicated *ad hoc* hypotheses In particular the following requirements must be fulfilled in the present case

A The model must be physiologically reasonable with respect to its structure

B The model must be compatible with the experimental radioiron data i e the plasma radioiron disappearance and the red cell radioiron appearance

C The model must generate acceptable pool sizes and flows i e acceptable from the point of view of other independantly obtained data on these quantities

Each of these requirements will now be discussed

Table 4 d

Flows and pool sizes from curve 2 when $J_{SC} = J$

T	g_1	P	S	J_{PS}	J_{SP}	J_{MP}	J_{PM}	M	J_S
	7 0	4 80	6000	27 3	2 56	30 6	5 9	64 1	24 7
	9 0	3 75	4804	25 7	2 37	30 9	7 6	61 3	23 3
3 0	11 0	3 00	3652	23 7	2 20	30 3	8 8	57 1	21 5
	13 0	2 70	3157	23 9	2 16	32 4	10 7	58 5	21 7
	15 0	2 25	2633	21 8	2 03	31 3	11 5	54 0	19 8
	7 0	4 80	864	27 3	3 84	29 1	5 6	41 2	23 5
	9 0	3 75	760	24 7	3 38	29 5	8 2	38 8	21 3
4 0	11 0	3 00	703	24 4	3 39	29 0	8 0	38 0	21 0
	13 0	2 70	693	24 6	3 43	31 1	9 9	39 7	21 2
	15 0	2 25	637	22 5	3 08	29 9	10 5	36 6	19 4
	7 0	4 80	375	28 2	6 00	24 5	2 3	20 9	22 2
	9 0	3 75	365	31 4	7 82	27 0	3 4	22 7	23 6
5 0	11 0	3 00	299	29 9	7 76	26 2	4 1	21 0	22 1
	13 0	2 70	276	30 1	7 41	27 8	5 1	20 8	22 7
	15 0	2 25	230	27 4	6 53	26 6	5 7	18 7	20 9

Table 5 a

Flows and pool sizes from curve 1 when $J_{MC} = J$

T	g_1	P	S	J_{PS}	J_{SP}	J_{MP}	J_{PM}	M	J_S
	7 0	4 80	911	4 00	4 00	28 8	28 8	172	44 9
	9 0	3 75	585	3 63	3 63	29 4	29 4	136	38 8
3 0	11 0	3 00	343	3 21	3 21	29 0	29 0	110	27 9
	13 0	2 70	280	3 16	3 16	31 1	31 1	99	24 9
	15 0	2 25	236	2 89	2 89	30 2	30 2	83	20 9
	7 0	4 80	91	9 12	9 12	23 5	23 5	121	57 2
	9 0	3 75	84	8 51	8 51	24 4	24 4	107	49 5
4 0	11 0	3 00	78	7 71	7 71	24 5	24 5	82	36 0
	13 0	2 70	82	7 02	7 02	27 3	27 3	58	31 8
	15 0	2 25	72	6 37	6 37	30 6	30 6	56	30 5
	7 0	4 80	98	11 7	11 7	21 1	21 1	115	99 5
	9 0	3 75	92	11 5	11 5	21 5	21 5	87	[illegible]3 2
5 0	11 0	3 00	83	11 0	11 0	21 1	21 1	67	56 0
	13 0	2 70	83	11 4	11 4	22 8	22 8	60	50 0
	15 0	2 25	75	10 9	10 9	22 1	22 1	50	41 2

5–20 mg/100 ml The total amount of non heme in the bone marrow can therefore be estimated to be of the order of 50–300 mg POLLYCOVE and MORTIMER (1961) using plasma radioiron disappearance data on a two compartment model with the assumption of red cell iron return to the plasma estimated the non heme iron pool in the bone marrow to be 50 to 150 mg Although these two estimates seem to be in good agreement it must be remembered that the chemically estimated pool is not necessarily identical with that estimated from isotope data The latter estimate refers to rapidly exchangeable iron whereas the former refers to all the iron present In view of the data obtained by FINCH (1959) on the exchangeability of body iron it appears unlikely that any substantial fraction of the bone marrow non heme iron is rapidly exchangeable It is also evident that the estimate obtained by POLLYCOVE and MORTIMER (1961) is critically dependant on the accuracy and precision of plasma radioiron data during the late part of the disappearance curve and that these characteristics are largely unknown As shown in the present work the pool size of M is not uniquely determined from the tracer data but that its calculation requires knowledge of the pathway of red cell iron return The smallest figure is obtained on the assumtion that no red cell iron is returned directly to the marrow In this case the present data indicate a pool size of the order of 17–70 mg the smaller values are obtained when T is large It is reasonable to assume that this estimated range is a maximum value for the size of the pool M since the incorporation into the model of a mechanism for ineffective erythropoiesis must always lead to a smaller estimation of the size of this pool

2 *The pathway of iron return from senescent red cells*

The question of the pathway of iron return from senescent red cells is of fundamental importance for the understanding of the whole system As can be seen from figure 1 the part played by the plasma iron pool in the total metabolism of iron will critically depend on whether the iron returns by way of the marrow iron pool and/or the precursor iron pool or whether the return is by way of the plasma pool and/or the storage pool In the latter case as much iron flows through the plasma as is being used for hemoglobin synthesis In the former case there is no net transport of iron through the plasma at all It is important to note that both these extreme possibilities are compatible with the plasma radioiron disappearance data The approach used in the present work permits some more definite conclusions on this point

Ad A In the absence of any detailed *a priori* knowledge concerning the basic structure of the system under investigation, we can only conclude that the model depicted in figure 1 is in accordance with generally held views The *assumption of pool homogeneity* cannot be supported by any other argument than the fact that the *tracer kinetics does not contradict it*

Ad B The experimental red cell radioiron appearance data and the initial part of the plasma radioiron disappearance data are in very good agreement with the model The generated values of the constants g, g_3 H_1, H_2 and H_3 which predict the pattern of the later part of the plasma radioiron disappearance curve are in good agreement with the data reported by POLLYCOVE and MORTIMER (1961) although it should be realized that *these figures are not* known with any high degree of accuracy or precision

Ad C This requirement is of special interest since it puts a definite upper limit to the total content of iron in the system i.e. the pool sizes of *S P* and *M* The sum of these is very unlikely to exceed a value of 2000 mg In fact the data of FINCH (1959) indicate strongly that total exchangeable non hemoglobin iron pool is less than 1000 mg In view of this restriction, the combination of $T = 3\ 0$ and the assumption of a quantitative iron return to the pool *S* can be rejected Of even more importance is the fact that the requirement C puts a very strong restrictive condition on one of the *flows namely the net hemoglobin synthesis iron flow* J_S since this is known with a considerable accuracy in the normal man The mean cell *life span in normal individuals is 122 days with* quite a small variation (BERLIN WALDMANN and WEISSMAN 1959 EERNISSE and VAN ROOD 1961, GARBY 1962) A red cell volume of 2000 ml thus predicts a daily net synthesis of hemoglobin iron of 19 mg To this estimate may possibly be added some 2 mg per day in order to account for normal "ineffective" erythropoiesis i.e. the formation of iron containing tetrapyrrols that will never *enter the circulation* or will stay there for very short time The present model generates values which are very close to this estimate for the flow J_S *in the cases (table 4)* where the return of iron from senescent red cells is to the plasma pool and/or the storage pool Furthermore there is *little variation in the value* of J_S when the constant g_1 varies within considerable limits

The size and significance of the pool *M* requires some comments Chemical estimations reviewed by HALLGREN (1953) indicate that the concentration of non heme iron in the bone marrow is of the order of

5–20 mg/100 ml The total amount of non heme in the bone marrow can therefore be estimated to be of the order of 50–300 mg POLLYCOVE and MORTIMER (1961) using plasma radioiron disappearance data on a two compartment model with the assumption of red cell iron return to the plasma estimated the non heme iron pool in the bone marrow to be 50 to 150 mg Although these two estimates seem to be in good agreement it must be remembered that the chemically estimated pool is not necessarily identical with that estimated from isotope data The latter estimate refers to rapidly exchangeable iron whereas the former refers to all the iron present In view of the data obtained by FINCH (1959) on the exchangeability of body iron it appears unlikely that any substantial fraction of the bone marrow non heme iron is rapidly exchangeable It is also evident that the estimate obtained by POLLYCOVE and MORTIMER (1961) is critically dependent on the accuracy and precision of plasma radioiron data during the late part of the disappearance curve and that these characteristics are largely unknown As shown in the present work the pool size of M is not uniquely determined from the tracer data but that its calculation requires knowledge of the pathway of red cell iron return The smallest figure is obtained on the assumtion that no red cell iron is returned directly to the marrow In this case the present data indicate a pool size of the order of 17–70 mg the smaller values are obtained when T is large It is reasonable to assume that this estimated range is a *maximum* value for the size of the pool M since the incorporation into the model of a mechanism for ineffective erythropoiesis must always lead to a smaller estimation of the size of this pool

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Although there has been no direct evidence for it, the usual assumption has been that all the iron used for hemoglobin synthesis flows through the plasma by way of the transferrin In the light of the present analysis this assumption is obviously equivalent to the assumption that there is no red cell death and subsequent iron transfer in the bone marrow i e $J_{MC} = J_{PC} = O$ The pathway of intravascular hemolysis and subsequent iron transfer has been excluded (GARBY and NOYES 1959) It has been claimed (MIESCHER 1956 EHRENSTEIN and LOCKNER 1958, see also BESSIS and BRETON GORIUS 1962) that the bone marrow is the chief site of destruction of red cells However the experimental data on which this claim is founded do not appear to be conclusive MIESCHER (1956) injected red cells labelled with Cr^{51} into rabbits and analyzed the bone marrow, the spleen and the liver for radioactivity at a time when most of the labelled cells had left the circulation i e after 25–35 days He found that a considerable part of the radioactivity was located in the bone marrow EHRENSTEIN and LOCKNER (1958) injected red cells labelled with Fe^{59} into rabbits and found "after a lapse of some hours , that a considerable part of the radioactivity lost from the circulation was found in the bone marrow The arguments for equating localisation of radioactivity with localisation of red cell death in these experiments are essentially kinetic and can be rejected on the same grounds since no information is given concerning the distribution kinetics of the labelled compounds whatever their nature *after* an initial localisation An attempt to correct for this complication was made by HUGHES JONES (1961 a and b) who concluded that red cell death in the bone marrow was probably significant in the rabbit but not in the rat BESSIS and BRETON GORIUS (1962) have given interesting evidence indicating that both erythrophagocytosis and ropheocytosis can occur in the bone marrow Their evidence however has no quantitative implications

The results obtained in the present study support the idea that only a small fraction of the red cells returns its iron directly to the bone marrow in human beings are admittedly indirect but seem to be the 5 a and b the notion of an iron return to these pools invariably gives too large values for the hemoglobin synthesis Furthermore the variability with respect to the flow J_S is unreasonably large These evidences against any significant direct red cell return to the bone marrow in human beings are admittedly indirect but seem to be the best existing

There are no experimental data to support either one of the two pathways J_{SC} or J_{PC} to be dominating This lack of knowledge makes

it impossible to estimate the pool size of S with any higher degree of accuracy. The results in the case when $T = 3.0$ and $J_{SC} = J$ offer some evidence against the idea that J_{SC} is the most important pathway since in this case the values for the pool size of S become unreasonably large.

3. *Hemoglobin synthesis as estimated from the initial plasma radioactivity disappearance curve and the plasma pool size*

It has been customary in clinical research to estimate the rate of hemoglobin synthesis, whether ineffective or not, by multiplying the *initial plasma radioactivity disappearance slope by the plasma pool size*. The estimate has been termed "plasma iron turnover". The work of SHARNEY, SCHWARTZ, WASSERMAN, PORT and LEAVITT (1954), HUFF and JUDD (1956) and POLLYCOVE and MORTIMER (1961) indicate that *such a procedure has no theoretical justification*: the plasma radioactivity disappearance curve does not exhibit the characteristics of a first order reaction. On the other hand, data concerning the "plasma iron turnover" in experiments designed to imitate increased and

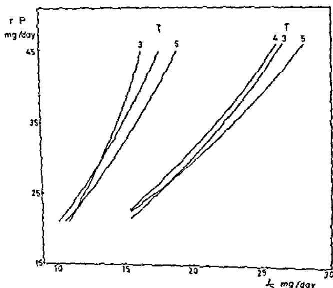


Figure 4. The relation between the "plasma iron turnover", $r \cdot P$, and the flow J_S in terms of the present model. The plasma pool size was set to 3.0 mg and the initial plasma radioactivity disappearance slope r varied between 7.0 and 15.0 per day. The curves to the left correspond to the red cell radioactivity appearance curve 1 and those to the right to curve 2.

decreased hemoglobin synthesis (BOTHWELL, HURTADO, DONOHUE and FINCH 1957) indicate that this estimate is indeed somehow related to the degree of erythroid marrow activity. In view of this fact it appears to be of interest to see how the "plasma iron turnover" correlates with the hemoglobin synthesis in terms of the model presented here. Figure 4 shows the relation between the two estimates in a series of cases calculated on the basis of the flow rate constants k of table 3 (a and b). As is seen the degree of covariation is quite large although the absolute values differ. It is evident that estimations of the absolute hemoglobin synthesis on the basis of the "plasma iron turnover" alone will not likely be very accurate. The difference between the two cases corresponding to the two different appearance curves is quite large. This point is illustrated also by the experimental fact (see table 1) that although the correlation between the product of r and the pool size P and the total red cell volume is quite strong (coefficient of correlation = 0.783), there is a not inconsiderable variation (s.e. of coeff. of corr. = 0.077).

4. *Concluding remarks*

The model presented and analyzed in the present work has been shown to be conceptually simple and to be consistent with the available experimental data. In addition to being a generalisation of the model of POLLYCOVE and MORTIMER its main characteristic and primary advantage is that it is based upon more data. Undoubtedly as more experimental data accumulate it will be subject to revision. The value of a model constructed as models are on insufficient data will be mainly judged by the success of experiments designed to disprove it.

One of the most critical points to be tested in the present system is that of the pathway of red cell iron return since direct estimations of the relative importance of the flow J_{SC}, J_{PC} and J_{MC} will allow more rigid conclusions.

Furthermore the presence of a labile bone marrow iron pool of the size predicted by the present model might be a fruitful approach of testing.

The details of the kinetics of iron uptake by the red cell precursors and their release into the circulation are additional predictions to be tested although it is interesting to note that different assumptions concerning the length of the time period T does not seem to influence the calculated value of J_e to any considerable degree (see tables 4 and 5 and figure 4).

The most obvious approach a precise evaluation of the later part of the plasma radioactivity disappearance curve does not seem to be possible at present since the radiation load would be too large

Summary

A model for ferro erythrokinetics in the normal human is presented

The quantitative evaluation of the model i e its flows and pool sizes is based upon a detailed analysis of the plasma radioactivity disappearance data and the red cell radioactivity appearance data as applied to the *a priori* given structure of the model

It is shown that the model is in accordance with the available experimental data In particular the generated values of the hemoglobin iron synthesis are in accordance with the data obtained from studies on red cell mean life span

The implications of the results are discussed and possible ways of testing the model are suggested

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I am grateful to Professor Dr med Johan Frimann Dahl for permission to use the facilities of the Radiology Department at Ullevål Hospital I moreover wish to thank my friend and colleague Dr Ragnar Hol for invaluable assistance in all experiments involving roentgenologic technique His interest and skill represent a major contribution to the investigation

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Contents

PART NOE

Introduction

The study of cardiovascular function has flourished greatly in the past two decades This progress has been strongly stimulated by the rapid development of instrumentation for the continuous measurement of pressure, flow and dynamic dimensional changes within the cardiovascular system

Hitherto most studies have covered a few mammalian species and have neglected the large variability in cardiovascular structure and function offered by the animal phylum

Our understanding of the cardiovascular function in lower vertebrate forms has hence become retarded compared to the concepts developed about cardiovascular regulation in a limited number of mammalian species

The present investigation is an attempt to study the dynamics of circulation in a lower vertebrate by using modern equipment for the measurement of intravascular pressures and pulsatile blood flow Additional information has been obtained from angiocardiographic analysis using high speed cinefluorography and film changer technique Efforts have been made to put the information acquired into a phylogenetical scheme The investigation has been divided into six parts

- Part One — Introduction material general anatomy methods and experimental procedures
- Part Two — Intravascular pressures in the intact free moving amphibian, *Amphiuma tridactylum*
- Part Three — A radiological study of the cardiac cycle in *Amphiuma tridactylum*
- Part Four — The functioning of the bulbus cordis in *Amphiuma tridactylum*
- Part Five — Cardiac output and dynamics of cardiac performance in *Amphiuma tridactylum*
- Part Six — An experimental evaluation of the double circulation in *Amphiuma tridactylum*

The objective of the present investigation is rather wide in scope and a clear definition and limitation of the problems pursued are not easy to reach owing to the prevailing ignorance about comparative cardio

vascular physiology in general In Part Two attempts are made to describe the course of venous and arterial pressures and the spontaneous reflex changes undergone in these in response to normal and artificial breathing and muscular activity

The sequential contraction and functional interrelationship between the various cardiac compartments in the lower vertebrate heart are but vaguely understood In Part Three, modern roentgenological instrumentation has been applied in an attempt to evaluate this problem in *Amphiuma tridactylum*

The lower vertebrate heart is characterized by the existence of the bulbus cordis as a separate chamber of the heart Part Four of this investigation is an attempt to describe the role this chamber plays in the dynamics of the heart Its importance in exerting a 'Windkessel' function with a distributing effect on the energy released by the contracting heart must be fundamental in animals with particularly low heart rates

The dynamic responses of the various cardiac compartments to different loads on the vascular system and their concerted action in the propulsion of the cardiac output have not earlier been subjected to experimental study in any lower vertebrate Part Five is a description of experiments designed to throw light on these problems

A main task of the cardiovascular system is the transport of oxygen from the respiratory organs to the site of respiration, the metabolizing body mass In lung breathing animals the success of this transport function is closely linked with the establishment of a double circulation, a separation of the pulmonary and systemic vascular circuits This separation is anatomically incomplete in lungfishes, amphibians, and reptiles The degree of functional separation between the two incompletely separated vascular circuits is a highly controversial issue, in particular with reference to the class *Amphibia*

In Part Six of the present investigation the results and equipment utilized in preceding parts have been used to design an experimental approach to the study of the pattern of circulation through the amphibian heart The degree of shunting between the circuits and the factors modifying the shunting pattern are investigated

MATERIAL

The species *Amphiuma tridactylum* (Cuvier) was selected as experimental animal in this investigation on account of several rare features among amphibians *Amphiuma tridactylum* is a comparatively large animal Adult specimens weigh between 600 1,000 g and measure from 70 100 cm in length This fact was of fundamental consequence as the experimental

procedures demanded access to smaller blood vessels for cannulation and sampling The lungs of *Amphiuma* are well developed and attain a considerable length The large lungs also made possible cannulation of the pulmonary artery and vein in the distal portions of the lung Pulmonary respiration is known to be the prime method for gas exchange (Baker 1949)

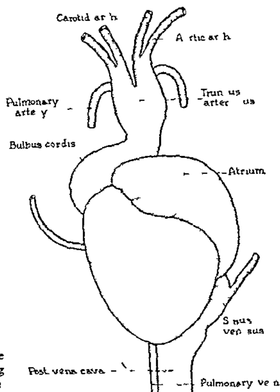


FIGURE ONE
Schematic drawing of the heart and larger adjoining vessels of *Amphiuma tridactylum*

The size of the bulbus cordis (Fig 1) permitted the use of electromagnetic flowmeter probes for measurement of pulsatile flow and cardiac output Differential measurements of pressure within the various heart cavities and in the pulmonary and aortic arches were likewise possible on account of the large size of these structures compared with conditions in other amphibians

The *Amphiuma* is readily anesthetized because of a proportionately large cutaneous and buccopharyngeal respiration The anesthetic is dissolved in the surrounding water and passes into the blood stream by diffusion *Amphiuma* is easy to keep and requires minimal attention in aquaria for extended periods of time

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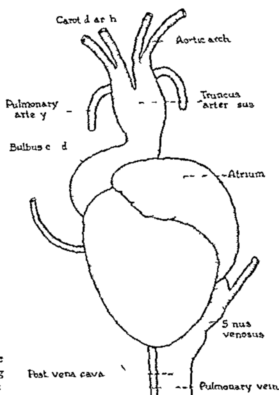


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From a phylogenetical viewpoint the urodeles in many respects form a nearly perfect transitional type between the fish and the land vertebrate, being in this regard much less specialized than the frog The transitional position is particularly noticeable in the anatomy of the vascular system, and it is surprising that there exists no adequate account of its functional characteristics

GENERAL ANATOMY OF THE CARDIOVASCULAR SYSTEM OF *Amphiuma tridactylum*

The literature on vertebrate anatomy is replete with references and descriptions of the cardiovascular system and its importance in ontogeny and phylogeny It is not the purpose here to make a thorough treatment of cardiovascular anatomy in *Amphiuma* Reference is only made to pertinent features in the physiological problems that are pursued in this investigation

Figure 1 depicts in a schematical way the heart and adjoining vessels The heart is situated distinctly to the right and slightly posterior to the coracoids It is enclosed in a pericardium which also encloses most of the truncus arteriosus The sinus venosus is an enlargement of the posterior vena cava in confluence with the right atrium In a ventral view it will normally be seen lying dorsally and to the left of the heart It joins the right atrium at the anterior, right hand, basal angle, quite close to where the right duct of Cuvier enters the sinus venosus Contralaterally the left duct of Cuvier enters at a basal angle The sinus venosus is a comparatively large chamber, capable of considerable distension The wall of the sinus venosus is very thin but contains quite distinct muscular elements The opening into the right atrium is guarded by a large flap valve, which prevents regurgitation of the blood from the right atrium to the sinus venosus (Züllich 1930)

The atria are markedly displaced to the left (Fig 1) From the outside it is impossible to distinguish any border between the right and left atrium The atrial wall is quite thin, with numerous trabecular muscle bands The inner surface is not smooth, and the numerous ridges and bands also form isolated smaller fingerlike cavities within the walls These detached projections give the atria a characteristic appearance It is apparent that the atria can accommodate fairly large volumes of blood Internal inspection reveals that the left atrium is considerably smaller than the right The atrial septum is very thin and consists of a delicate muscular network, the fibers of which are continuous with those of both atria

The single ventricle, like that of most amphibians, is composed of a trabecular muscular network, so dense that it gives the appearance of a

muscular sponge with a small central cavity The ventricular lumen is thus in essence composed of a multitude of very small compartments like the meshes of a sponge The trabecular muscle bands show a tendency to run in an antero posterior course The blood will in many places penetrate close to the surface of the ventricle, where it may be observed through the semi transparent wall The ventricular myocardium is nourished by the blood in the chamber and is devoid of a coronary supply In *Amphiuma* the central ventricular cavity is very small, consisting of an L shaped lumen extending from the atrioventricular opening to the bulbus cordis Regurgitation to the atria is prevented by a pair of flap valves

Between the ventricle and the arterial trunks are interposed the bulbus cordis and truncus arteriosus (Fig 1) The bulbus cordis is a chamber in direct confluence with the ventricular myocardium and hence contains striated muscle cells whereas the more distal truncus arteriosus contains smooth musculature similar to that found in vascular walls in general The bulbus cordis arises from the right antero ventral angle of the ventricle and is inclined to the left It is cylindrical at its junction with the ventricle but expands transversely as it proceeds cranially into the truncus arteriosus Reflux of blood from the bulbus into the ventricle is prevented by the ventriculo bulbar valves consisting of three non muscular semilunar cusps The border between the bulbus cordis and the truncus arterio us is similarly guarded by four semilunar valves, one of which prolonged caudally is the spiral valve with its ventral edge lying free At its proximal end the spiral valve is attached to the left bulbus wall, later passing obliquely across the bulbus and ending with a spiral twist back to the left Just cephalad to this spiral twist is a ridge separating the upper part of the bulbus into two channels The right channel connects with the two parts of systemic arteries in the truncus arteriosus the carotid and aortic arches while the left channel opens into the two divided pulmonary arteries

The truncus arteriosus represents a base for the arterial arches These however are all separated along the entire truncus in *Amphiuma* although the structure is enclosed in a sheet which grows forward and around it from the bulbus The greater vessels originating from the truncus arteriosus are the carotid aortic, and pulmonary arches The aortic arches pass to the branchial region where they loop behind and around the branchial fissure They join to form the single dorsal aorta in posterior direction The pulmonary arches turn caudal abruptly and pass along the lateral side of the lungs There is no ductus Botalli

The equipment used for the measurements of intracardiac and intravascular pressures consisted of Statham pressure transducers (model P23BB) and a differential pressure transducer (model Sanborn 267B) The transducers were connected with Sanborn carrier pre amplifiers and a 4 channel Sanborn recorder The transducers were calibrated against columns of distilled water During actual recording the transducers were connected with the various measuring sites by polyethylene catheters, *according to the detailed description below The flowmeter used (Parts* Four and Five) was of the electro magnetic type, produced and constructed by Kiger Dennard Associates, U S A

Calibration of the flowmeter was done postmortally with the probe in the same position as during actual recording A short tube was inserted through the ventricular wall and passed on to the proximal end of the bulbus, where it was tied in place Known volumes of blood or Ringer *solution were injected into the tube and passed through the flow probe* at the distal end of the bulbus cordis The deflection obtained on the flowmeter record permitted calculation of the actual flow in ml per time unit

The equipment utilized in the radiological experiments (Parts Three and Six) consisted of an under couch tube and a Philips image intensifier connected with an Arriflex camera which permitted rate of exposures limited upwards to 30 frames per second In other experiments an Elema Schonander film changer was used This equipment utilizes 24 x 20 cm film and gives a high degree of resolution The rate of exposures varied between 2-4 frames per second, and they were made at 50 54 KV and 200 mA Exposure time 0 02—0 04 sec Film focus distance 90 cm Tube focus 1 2 x 1 2 mm

A total of 75 specimens of *Amphiuma tridactylum* were used in the investigation

The animals were anesthetized by total immersion in Ms222[1] at a concentration of 5 g/l During the immersion period the animals were deprived of access to atmospheric air, and anesthesia occurred in 10 25 minutes, depending upon the size of the animal

Fifteen specimens were used *in Part Two of the* investigation The experimental procedures were as follows An incision was made in the midline ventrally, 10 15 cm cephalad to the cloacal opening The pulmonary artery was dissected free and cannulated with a polyethylene catheter (P E 10) The cannulation was performed in the distal end of the lung At this level the diameter of the pulmonary artery reaches

[1] Tricaine Methanesulphonate produced by Sandoz Switzerland

maximally 0 5 mm A small artery branch of the gut was usually chosen for cannulation to obtain the systemic blood pressure During cannulation the catheter tip was pushed toward the dorsal aorta, often projecting into the lumen of the latter vessel A small vein in the mesenterial lining was cannulated in a similar way for recording of venous pressures In all these cannulations polyethylene catheters were used and the vessels tied around the catheters The smallest vessels possible for cannulation were used and no consequential changes in blood flow and distribution were likely to ensue Subsequent to cannulation the catheters were filled with heparinized Ringer solution and closed at the end In some experiments the tip of one lung was cannulated making possible record ings of an interference with the intrapulmonary pressures The incision in the body wall was sutured in a double layer, special care being taken to close the lining of the visceral cavity and the muscle layer The various catheters were marked and guided out through the caudal end of the incision The animal was transferred back to the aquarium and within a very short time woke up and moved about seemingly undisturbed These indwelling catheters could be in place for 8-10 days Pressure recordings were made by connecting the implanted catheters to pressure transducers via connecting catheters No recordings of pressures were made before the animals had completely recovered from the anesthesia

Fifteen large specimens weighing from 800 900 g were used for the experiments described in Part Three The operative procedures were kept at a minimum allowing only for cannulation of the posterior vena cava or the common pulmonary vein The water soluble contrast medium Hypaque 45% was injected through polyethylene catheters (P E 10 P E 50) The injection volumes never exceeded 2 ml The radiological examinations were made with the animals placed in both dorsal and ventral positions Additional side projections with a *horizontal beam* direction were done to check the extent of contrast sedimentation

The experiments described in Parts Four and Five were done on 20 specimens After induction of anesthesia a small incision was made at a level corresponding to the distal end of the left lung (10 cm cephalad *to the cloaca*) The tip of one lung was exposed and cannulated with a polyvinyl catheter This cannulation gave access to inflation and ventila tion of the lungs with selected gas mixtures During light anesthesia spontaneous breathing usually persisted

Another incision was made in the midventral line from a level corre sponding to the front legs extending to a level 2 cm behind the heart The contracting heart can be observed as bulgings on the skin Careful dissection exposed the systemic and pulmonary arterial arches Opening of the pericardium gave free access to the heart structures, including the

bulbus segment Access to the posterior vena cava was secured close to the heart

Pressures from the arterial arches, the bulbus cordis and other cardiac compartments were recorded by introducing a needle catheter of a proper size through the vessel wall in an upstream direction or directly through the walls of the cardiac chambers The needle catheters were made from polyethylene tubing with the tip of a hypodermic needle inserted at the distal end The tubings used ranged between P E 10, 20, and 50, with needle tips of gauge 30, 27, and 20, according to the size of the vessel to be cannulated The introduction of a needle catheter presumably caused no significant obstruction to the blood stream The length of the catheters was standardized, and their size always matched in experiments of the same kind Four pressures could be recorded simultaneously

A square wave electro magnetic flowmeter was used for measurement of stroke volume and cardiac output The flow probes used were care fully selected to fit the vessel snugly without exerting unnecessary pressure on the vessel or the adjoining tissues Flow was most commonly measured in the distal portion of the bulbus cordis, where the rhythmic changes in size with each heartbeat are smallest

Part Six of the investigation is based upon studies of 25 specimens In the experiments involving oxygen analysis and pressure measurements, the heart and adjoining vessels were exposed and dissected free, precau tions being taken to eliminate hemorrhage

Blood samples for oxygen analysis were collected simultaneously from the pulmonary artery and an aortic arch, followed by immediate sampling from the sinus venosus and pulmonary vein The sampling was done by inserting a 30 gauge cannula through the appropriate vessel The cannula was connected by a small plastic adapter directly on to a micro pipette used in the analysis of the blood gas The small volume withdrawn (25 μl) was anaerobically transferred to the blood gas analyser for analysis by the method of Scholander and van Dam (1956), or with the use of a Natelson micro blood gas analyser In most experiments the lungs were inflated with pure oxygen some time prior to an experiment The skin was kept moist to maintain normal cutaneous respiration

The large size of the experimental animals permitted repeated sampling without disturbance of the blood volume and intravascular pressures The radiological experiments followed the procedure described in Part Three, and the intravascular pressure measurements followed the proce dure described in experiments in Parts Four and Five

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PART TWO

Intravascular pressures in the intact, free-moving amphibian, *Amphiuma tridactylum*

Recent studies on mammalian species have demonstrated a marked difference in the cardiovascular characteristics of the nonanesthetized, unrestrained subject compared to the anesthetized animal These findings have necessitated modifications in earlier concepts of cardio vascular regulation and control (Rushmer *et al* 1960)

Attempts were made in these experiments to record cardiovascular parameters in the conscious, unrestrained amphibian, *Amphiuma tridac tylum* The interrelations of breathing patterns and muscular movements to cardiovascular responses were examined

In the urodeles five methods of respiration are utilized (Noble 1925), namely branchial, pulmonary, cutaneous, aerial buccopharyngeal, and aquatic buccopharyngeal respiration Modifications in development tend to eliminate certain of these methods, but most often the animals special ize and one or two of the methods will prevail In *Amphiuma*, which is an aquatic amphibian, the four last methods are undoubtedly in operation, although pulmonary respiration is the most important (Baker 1945)

RESULTS

Figure 2 demonstrates an arterial blood pressure tracing recorded through an indwelling catheter The animal was submerged and rested quietly in the aquarium The systolic pressure fluctuated in a cyclic pattern around 30 mm Hg The corresponding diastolic pressure was

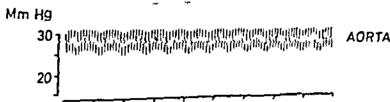


FIGURE TWO
Blood pressure record from the aorta of an intact free moving specimen of *Amphiuma tridactylum* Note the cyclic changes in blood pressure Time marks 30 seconds

about 25 mm Hg giving a pulse pressure of 5 mm Hg The blood pressure cycles consisted of regularly recurring cycles of about 20 sec duration (Fig 2) The cycles were not related in any way to the respiratory movements or breathing pattern otherwise They were not consistently present but occurred when the animals rested quietly for fairly long periods

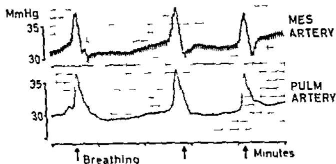


FIGURE THREE

Demonstration of the marked pressure increase in both the systemic and pulmonary circuit that occurs with each respiratory effort Time marks one minute

Figure 3 shows simultaneous recordings of arterial pressure from a mesenteric artery branch and from a pulmonary artery cannulated at the tip of the lung The difference in pulse pressure can be ascribed to the marked difference in distance from the cannulating sites to the heart The small pulse pressure in most tracings in Part Two results from large damping inherent in the small bore catheters used to obtain indwelling cannulation of small vessels A sharp rise in pressure occurred with each breathing cycle The animal lifted its head about 1 2 cm above water level inhaled by lowering the floor of the mouth, and performed a series of swallowing movements During the breathing effort there was a noticeable increase in the muscle tension in the body wall

It was demonstrable in all experiments with intact animals that the state of contraction of the musculature in the body wall had a decisive influence on the intravascular pressures The diastolic level of pressure in the pulmonary artery often showed abrupt, spontaneous changes probably of a vasomotor origin

Figure 4 shows fluctuations in arterial and venous pressure during a period af active movement The fluctuations in the intravascular pressures were obviously related to the muscular activity

The following is a brief description of some spontaneously occurring changes in blood pressure and heart rate of probable reflex origin Some attempts to evoke such reflex changes in blood pressure by artificial means are described

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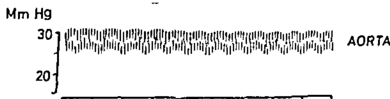


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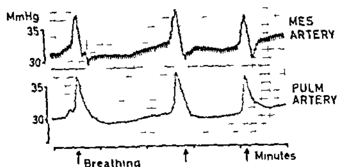


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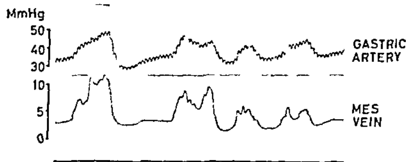


FIGURE FOUR
Fluctuations in arterial and venous pressure during a period of active movement
Time marks 30 seconds

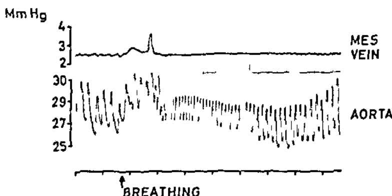


FIGURE FIVE
Venous and arterial pressures recorded in an intact specimen of *Amphiuma tridactylum* A marked tachycardia resulted subsequent to a pressure rise following a breathing movement
Time marks 15 seconds

Figure 5 shows recordings of venous and arterial pressure At the point of the arrow the animal lifted its head above water and commenced an inspiration The initial response consisted of a sudden pressure increase in the artery and the vein, immediately followed by a marked tachycardia The frequency became doubled and the pulse pressure reduced accordingly Gradually the heart rate returned to initial values, while the pulse pressure simultaneously increased There was also a distinct pulse noticeable in the venous pressure record Figure 6 similarly demonstrates a reflex doubling of the heart rate at the first inspiration This time, however, another inspiration took place before the heart had returned to its initial frequency In spite of a similar change in pressure, which was obviously related to the increased muscle tension, no further increase in heart rate was detectable at the second inspiration The reflex change in heart rate concurrent with the inspiratory effort was obviously dependent upon the initial heart rate In an attempt to determine the effective stimulus to the reflex tachycardia described in Figures 5 and 6

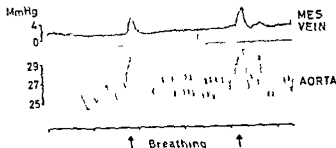


FIGURE SIX
Venous and arterial pressure recorded during two successive breathing movements In response to the first a marked tachycardia resulted The frequency remained unchanged after the second breathing movement Time marks 15 seconds

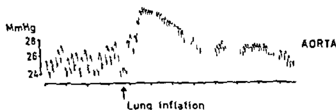


FIGURE SEVEN
Aortic pressure recorded during an artificial inflation of the lungs Note the pressure rise and subsequent tachycardia Time marks 15 seconds

an artificial inflation of the lungs through an indwelling catheter was performed Care was exercised to inflate the lungs to a natural degree of distension The inflation evoked no signs of discomfort or abnormal behavior in the animal Figure 7 demonstrates the marked tachycardia that resulted from inflation of the lungs Again the tachycardia was dependent upon the initial heart frequency and rarely occurred if a high rate prevailed when the stimulus was applied In all experiments giving a reflex onset of tachycardia the initial frequency was below 20 beats per minute

A reflex tachycardia could similarly be elicited by other stimuli Figure 8 was recorded when the water surrounding an animal was slowly drained from the aquarium At the time indicated by the arrow the water level had sunk to a point where the dorsal side of the animal was exposed to air A sudden contraction of the body musculature could be observed The arterial pressure as well as the heart rate rose immediately

Typical depressor responses were similarly observed to occur spontaneously in *Amphiuma* Figure 9 demonstrates arterial and venous pressures as well as pressure inside the lung, recorded continuously in an intact

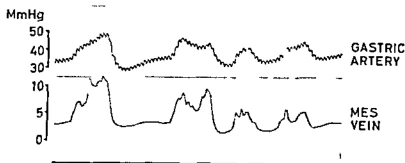


FIGURE FOUR

Fluctuations in arterial and venous pressure during a period of active movement Time marks 30 seconds

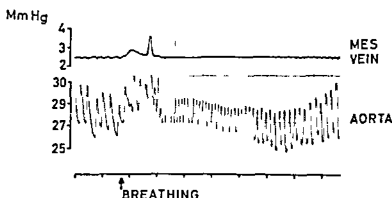


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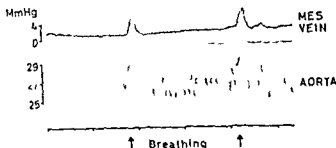


FIGURE SIX

Venous and arterial pressure recorded during two successive breathing movements. In response to the first a marked tachycardia resulted. The frequency remained unchanged after the second breathing movement. Time marks 15 seconds

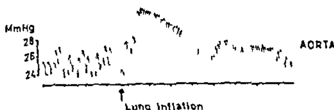


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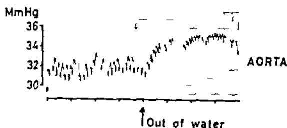


FIGURE EIGHT

Aortic pressure recorded during a slow drainage of the water in the aquarium At the time of the arrow the water had receded and the animal responded with a general muscle contraction Time marks 15 seconds.

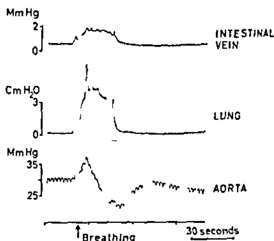


FIGURE NINE

Simultaneous recordings of venous intrapulmonary and aortic pressures A spontaneously occurring breathing movement gave a rise in all pressures followed by a typical depressor response in the aortic pressure record Time marks 30 seconds

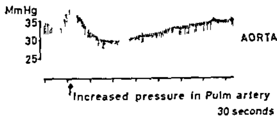


FIGURE TEN

Aortic pressure showing a typical depressor effect in response to an artificial increase of pressure in one pulmonary artery Time marks 30 seconds

animal At the time shown by the arrow an inspiration started The venous pressure rose and stayed elevated during the entire breathing process The pressure inside the lung increased from 0 to 3 cm of water, demonstrating the typical positive pressure breathing in amphibians The arterial pressure rose sharply but subsequently fell abruptly toward a minimal value, after which it climbed to initial pressure levels This typical depressor response could also be elicited in the intact animal by artificially increasing the pressure in the pulmonary artery while recording the systemic arterial pressure, or vice versa Figure 10 depicts the common response to such a procedure, demonstrating the presence of a depressor mechanism

Small amounts of epinephrine were injected intravenously in the resting, awake animal Figure 11A shows a marked increase in the force of contraction apparent in a higher diastolic pressure and an increased pulse pressure In the experiment underlying Figure 11B, the response was in addition associated with a conspicuous tachycardia

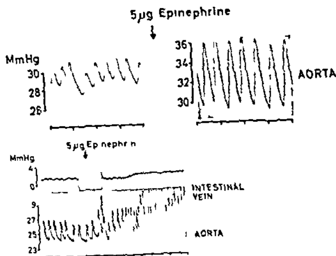


FIGURE ELEVEN
Response in arterial and venous pressures to intravenous injection of 5 µg epinephrine Time marks 6 seconds

DISCUSSION

The author is unaware of any published study of a similar nature using intact nonanesthetized species of amphibians as experimental subjects Earlier references to intra arterial pressure measurements in urodeles are also entirely lacking Schulz (1906) recorded values up to 60 mm Hg for *Rana esculenta* The pressure values obtained in this study compare with those recorded by de Graaf (1957) for the anuran *Xenopus laevis* Similarly, the systolic values of pressure correspond with those obtained by Shannon and Wiggers (1939) in the frog (*Rana sp*) Kuno (1914) recorded an average blood pressure of 30 mm Hg in large specimens (200 g) of *Rana esculenta* More recently Simons (1957) has presented a paper on the blood pressure and pressure pulses in the arterial arches of the frog and the toad The pressures in the pulmocutaneous vessels were on the average the same as those in the systemic and carotid arches These averaged 26/19 mm Hg (systolic/diastolic) in the toad against 15/8 mm Hg in the frog

Rhythmically recurring cycles in blood pressure of a similar nature to those depicted in Figure 2 have been described in the frog by Kuno (1914) The changes he recorded were not related to changes in frequency of respiration or pulse pres ure changes Hence he attributes the changes to cyclic differences in the tonus of smooth musculature of the vas cular walls He feels justified in comparing them with the blood pressure

cycles described by Mayer (1876) in the mammal A rhythmic vasomotion resulting in blood pressure cycles had already been described in the frog by Saviotti (1870) Similarly Riegel (1871) observed rhythmical contractions of the mesenteric arteries of frogs

The lability in the levels of intravascular pressures in intact *Amphiuma* apparent with each respiration or the slightest movement has an obvious relationship to the state of muscular contraction In anesthetized frogs and toads Simons (1957) recorded small changes in the pulmonary arterial pressure levels seemingly without corresponding changes in systemic arterial pressure Simons feels justified in attributing these changes to the degree of inflation of the lungs which will directly affect the pressures registered in the pulmo cutaneous arteries The cylindrical elongated body form of *Amphiuma* with all the visceral structures enclosed within a fluid filled cavity surrounded by a muscular body wall is immediately suggestive of a relationship between muscle tension and intravascular pressures An increased muscle tension will affect arterial as well as venous pressures directly, which in turn can give secondary reflexly induced pressure changes The net effect of such interrupted pressure elevations transmitted from increased muscle tension (Figs 3, 4, 8) will undoubtedly be a facilitation of the cardiovascular function In particular the vascular capacity spaces, i e the larger veins, will empty their contents and increase the venous return quite markedly This in turn will augment cardiac contraction and output

Most of the available knowledge on cardiovascular reflexes is of necessity derived from experiments with anesthetized, surgically treated animals The quality and strength of the nervous and mechanical stimuli which elicit these reflex responses in experiments may not have a parallel in any normal situation which the animal is likely to encounter Such experiments may elucidate the mechanisms of the reflex action, but fall short of explaining their normal role in physiological adjustment

The results obtained in the present study are a clear manifestation that cardiovascular reflex responses can be easily evoked in *Amphiuma* and, even more important, that the reflexes occur within the limits of stimulus threshold that are encountered in experimental situations as close to normal as possible We are therefore justified in interpreting both depressor responses and reflex changes in heart rate as normally occurring physiological adjustments in the intact *Amphiuma* Since the exact nature and site of action of the stimuli are not known, the data obtained permit no analysis of specific reflexogenic areas of the vascular system of *Amphiuma* The reflex onset of tachycardia occurring spontaneously (Figs 5, 6, 7, and 8) might have been the result of a stimulation arising from an increased venous return accompanying the increase in general

body muscle tonus Its dependence upon the initial frequency was markedly consistent Particular interest attaches to the tachycardia following either a normally occurring inspiration or an artificial inflation of the lungs (Figs 6 7) One cannot exclude the possibility that this effect results from a direct stimulation of inflation receptors in the lungs In particular the experiments with artificial inflation point in that direction since no accompanying muscular effort increased the venous pressure and return of blood to the heart Hering (1871) is credited with being the first to observe that an increase in the pulmonary inflation volume causes an acceleration of the heart Anrep *et al* (1936) showed that the heart rate changes associated with lung inflation are of central as well as of reflex nature A moderate inflation such as a spontaneous inspiration causes a cardiac acceleration whereas a stronger inflation causes a bradycardia Von Saalfeld (1933) independently arrived at the same results Anrep *et al* worked with a heart lung preparation, whereas von Saalfeld used an intact preparation but artificial ventilation More recently Aviado and Schmidt (1955) have emphasized the generally accepted view that reflex circulatory effects of normal lung inflation and deflation are insignificant in man Simons (1957) raises the possibility that nervous control mechanisms responding to lung inflation may exist in frogs and points to the observation of Neil, Strom and Zotterman (1950) that afferent fibers of the vagus originating in the lung tissue show an increase in impulse discharge as the lungs are inflated The cardiac acceleration following spontaneous inspiration in *Amphiuma* may be of importance in showing that the effect is a normally occurring phenomenon with a possible regulatory function on heart rate

The depressor responses recorded (Figs 9 and 10) similarly cannot be attributed to specific stimulator sites especially as there is anatomical connection between the systemic and pulmonary circuits in all *Amphibia* A pressure increase applied in the pulmonary artery may well exert its stimulatory effect in the systemic circuit and vice versa

The presence of a depressor mechanism in the arcus aortae of amphibians (*Rana*) was demonstrated by Kuno (1914) He increased the stretch of the aortic wall by ligating the contralateral aortic arch which resulted in a prompt decrease in blood pressure after a slight initial rise He also clearly showed that electrical stimulation of the central end of the cut vagus caused a reflex fall of blood pressure On more uncertain ground Nikiforowsky (1913) claimed that there are depressor afferents in the vagus of the frog However, Neil Strom and Zotterman (1950) searched in vain for vagal nerve fibers connected to the main vessels of the truncus arteriosus They suggest that the afferent fibers stimulated by Kuno arise directly from the heart and not from the aorta This however opposes the

finding that increased aortic pressure leads to a blood pressure fall The presence of sensory innervation of the truncus arteriosus has not yet been demonstrated Meyer (1927) demonstrated tonically active depressor fibers in the glossopharyngeal nerve of the frog by local increase in intravascular pressure in the carotid region he could evoke a depressor effect He concluded that this vascular reflex must be homologous to the carotid sinus reflex first described by Hering (1924, a,b) in the mammal Neil, Strom and Zotterman (1950) showed that the proper nerve endings in the frog could be activated by stretching the blood vessels The nerve endings seemed to arise from the carotid gland as well as from the adjacent part of the common carotid artery Carman (1955) rejected the idea that the carotid gland of amphibians is associated mainly or only with vaso- and possibly chemoreceptor functions Its structure indicates some distributing adjustment of blood flow in the external carotid artery, since this vessel bifurcates at an acute angle to the common carotid It should be noted that many amphibians, among these *Amphiuma*, lack a carotid gland It seems likely that the phylogenetical development of the carotid sinus reflexes as they appear in the mammal has more diffuse origin Such a viewpoint receives support from the widespread sensory areas in the branchial system found in the lowest vertebrates The carotid arteries of the higher vertebrates are true derivatives of the primitive branchial system of the lower vertebrates Lutz and Wyman (1932) and Satchell (1961), among a number of others, have amply demonstrated the unspecific origin of inhibiting cardiovascular reflexes in the lower vertebrates, notably the elasmobranchs

Lutz and Wyman (1932) were able to obtain cardiovascular reflex responses in the urodele *Necturus maculosus* With this exception, there are no reports on reflex cardiovascular responses in other urodele amphibians

It seems noteworthy that the reflex response depicted in Figure 10 may depend on baroreceptors located in the pulmonary circuit The existence of vascular baroreceptors in the pulmonary vessels has been claimed from time to time Churchill and Cope (1929) and later Schwiegk (1935) proved that the bradycardia and hypotension resulting from increased pressure in the lung circuit are of reflex nature and depend on the integrity of the vagi Aviado *et al* (1951) and Guyton *et al* (1954) have more recently claimed that baroreceptors localized in the pulmonary arteries of mammals exert a significant inhibitory effect on the vasomotor center Other authors, Daly, Ludany, Todd and Verney (1937) have produced evidence that the pulmonary veins possess a sensory innervation linked with cardiovascular and respiratory reflexes in mammals No reports on the existence of reflexogenic areas in the pulmonary vessels of lower vertebrates have come to my attention The

response depicted in Figure 10 may have resulted from stimulation of receptors located to the venous side of the pulmonary circuit In this connection it should be noted that the caliber of capillaries in *Amphiuma* in all likelihood surpasses that known in other vertebrates The red cells of *Amphiuma* are 70 80 μ in diameter and by far the largest on record Hence an artificially applied pressure head on the arterial side will also immediately exert an increase in pressure on the venous side of the capillary bed

The effects of intravenous infusion of epinephrine in the intact *Amphiuma* are demonstrated in Figure 11 With reference to Figure 11B it is particularly noteworthy that the increase in venous pressure precedes the onset of a rapid pressure rise in the aorta In this connection it is an important but often overlooked fact that epinephrine constricts veins This observation was noted by Oliver as long ago as 1897 By this act a considerable effect on the capacity of the vascular system is exerted McDowall (1935) has emphasized that the main role of the sino-aortic reflexes is the adjustment of the circulatory capacity, thus influencing the cardiac output and general systemic blood pressure

SUMMARY

Intravascular pressures have been recorded in intact nonanesthetized specimens of the large salamander *Amphiuma tridactylum* The systolic values of arterial blood pressure fluctuated around 30 mm Hg with a pulse pressure of about 5 mm Hg The level of blood pressure was related to the state of activity of the animal Movements and respiratory efforts were commonly associated with increased pressure

Some spontaneously occurring reflex changes in heart rate and blood pressure are described A reflex tachycardia was observed in response to inspiratory efforts A similar tachycardia could result from an artificial inflation of the lungs or from a sudden contraction of the body wall musculature The tachycardia response depended upon the initial heart frequency Typical depressor responses were also observed to occur spontaneously The phylogenetical relationship of a branchiogenic intravascular baroreceptor area is discussed

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PART THREE

A radiological study of the cardiac cycle in *Amphiuma tridactylum*

Cinefluorography and high speed film changer techniques are particularly well suited for analysis of the cardiac cycle with regard to functional anatomy, timing of the contractile processes, and other hemodynamic events Information of this kind is scarce for most lower vertebrate forms, and the author is unaware of any such study having been done on an amphibian

The following is a survey of results obtained with both the above methods on lightly anesthetized specimens of the large amphibian *Amphiuma tridactylum*

RESULTS

The contrasts injected in the posterior vena cava outlined the inflow channels to the right atrium clearly As it approaches the heart, the posterior vena cava is discernible as a large vessel slightly to the left of the midline (Fig 12A) The vessel is in direct confluence with the sinus venosus and no valves demarcate the beginning of the latter A functional border between the posterior vena cava and the sinus venosus became apparent when the latter exhibited rhythmic contractions These contractions could be observed as a general shortening of the sinus venosus as well as a narrowing of the vessel diameter The reduction in diameter never resulted in a closure of the vessel lumen Accordingly, one would assume that a certain regurgitation into the greater veins takes place as the sinus venosus contracts The contractions resulted in a reduction to about one half of the maximal area covered by the contrast filled sinus venosus (Fig 12 B, D) The main chamber of the sinus venosus has a triangular shape It is a capacity space and shows ordinarily a considerable residual volume after filling of the right atrium (Fig 13)

The two other inflow channels to the sinus venosus, the right and left ducts of Cuvier, are relatively small and enter the sinus venosus at acute angles in the anterior direction There are no valves preventing regurgitation into these vessels

The inflow tract from the sinus venosus to the right atrium has a

characteristic appearance (Fig 12) In its posterior part, shortly anterior to the entrance of the left jugular vein there is a narrowing giving the sino atrial connection the shape of a small dilatation The sino atrial valves are most likely situated at the point of this narrowing At the time of closure of these valves the sino atrial connection was interrupted right at this point (Fig 12B) Following opening of the sino atrial valves the initial filling took place while the sinus venosus volume was still increasing and may therefore be attributed to the prevailing pressure head in the larger veins Shortly later, the sinus venosus started to contract actively, and seemed responsible for a significant portion of the right atrial filling

At a heart rate of 30 beats per minute with a cardiac cycle length of 2 seconds the sinus venosus displayed volume changes consisting of a rapid distension followed by a more extended subsequent contraction (Fig 12 A, B, C D) The timing of the sinus venosus contraction and its relation to the other contractile processes of the heart are revealed in Figure 14 This Figure is a plot of the changes the various cardiac chambers undergo throughout the cardiac cycle measured as projected areas on the films The films supporting the analysis were exposed at a rate of 4 frames per second Owing to the limited rate of exposures (four frames per second) the accuracy in the timing of the minimum and maximum values in the areas covered by the various cardiac compart

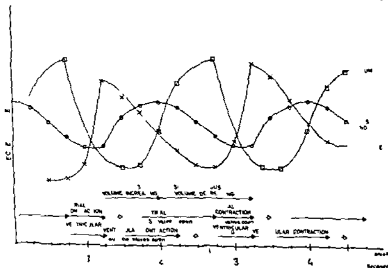


FIGURE FOURTEEN

Demonstration of the interrelationship between the various cardiac compartments measured as projected areas on the roentgen films throughout a cardiac cycle At the bottom of the Figure is indicated the time sequence of the filling and contraction of the various compartments The films on which the Figure is based were exposed at a rate of 4 frames per second at 50 Kv Exposure time 0 04 seconds

ments, and hence the timing of opening and closure of the valves, will be within 0.25 seconds, as indicated by the stippled lines in the lower part of the Figure.

Atrial filling (Fig. 14) lasted 1 second at a heart rate of thirty beats per minute. After the sino-atrial valves had opened there was a rapid phase of atrial inflow while the sinus venosus volume was still increasing. The early rapid filling lasted for about 0.25 seconds and contributed approximately 1/3 of the atrial filling, judged from the area projected on the roentgen films. The rapid influx of blood to the atrium was sustained for another 0.25 seconds, after which the inflow rate declined and reached a summit 0.5 seconds later. These latter phases of atrial filling were presumably a consequence of an active contraction of the sinus venosus. This was observed by direct visual inspection as well as on the roentgen films, and was verified by intracardiac pressure measurements (Part Five).

The right atrium had a characteristic appearance. It showed a central cavity, which received the inflow from the sinus venosus. This central lumen had connections to a number of peripheral projections, which were subsequently filled (Fig. 12A). At times when the volume flow through the heart was low, the fingerlike projections were not filled at all. However, as soon as the venous return increased, for instance by a slight lifting of the tail, the atrial filling involved the extensive system of diverticula. The fact that the right atrium adjusted its effective lumen according to the volume inflow by filling of these diverticular spaces may have consequences for its performance (see Discussion, p. 65). The characteristic structure of the right atrium suggests that it forms a capacity space. In fact the right atrium could accommodate a larger volume than any of the other cardiac chambers.

Atrial contraction turned out to be a remarkably rapid process. The atrio-ventricular valves opened in response to this contraction and the entire process of ventricular filling was the result of atrial contraction. One could substantiate this observation by direct visual inspection. No blood entered the transparent ventricle prior to atrial contraction. The right atrio ventricular connection was situated slightly median to the sino atrial connection. The direction of inflow was in antero posterior direction, making an initial 60° angle with the midline and entering the ventricle at its left medio lateral side. The atrio ventricular valves were open 0.5 seconds or 1/4 of the cardiac cycle (Fig. 14). The right atrio ventricular opening was of a large caliber, no doubt of importance for the rapid ventricular filling. Most commonly there was a clear preference for selective filling to the right portion of the ventricle when the contrast entered through the right atrium. The selective filling was clearly supported by the numerous muscular ridges and trabeculae inside the ven

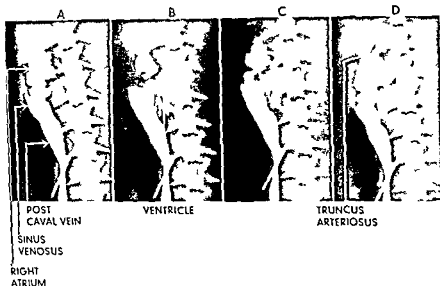


FIGURE TWELVE

Contrast injection in the posterior vena cava Animal in ventral position Rate of exposure 2 frames per second at 50 Kv Exposure time 0 03 seconds A B C D are consecutive frames

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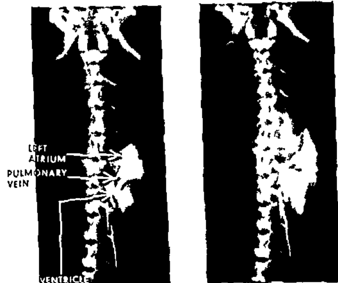


FIGURE FIFTEEN

Contrast injection in the pulmonary vein Animal in dorsal position Rate of exposure 2 frames per second at 50 Kv Exposure time 0 03 seconds Frames A and B are spaced one second apart

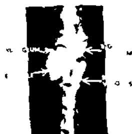


FIGURE SIXTEEN

A contrast injection in the posterior vena cava has outlined the sinus *venosus* the right atrium and the ventr cl The first set of bulbar outflow valves is seen situated quite far up into the bulbus cordis Exposure done at 50 Kv Exposure time 0 04 seconds



FIGURE THIRTEEN

Maximum and minimum area covered by the sinus venosus Rate of exposure 4 frames per second at 52 Kv Exposure time 0 04 seconds Frames A and B are spaced one second apart

tricle The inflow pattern to the ventricle had a very characteristic appearance The filling occurred in discrete furrows demonstrating the dense trabeculation of the inner ventricular surface This trabeculation was arranged mainly in antero-posterior direction The lumen of the ventricle was thus split into numerous slits, in structural confluence but with a seemingly high degree of functional separation The atrial residual volume showed great variance

The left atrium is smaller than the right It is filled directly from the pulmonary vein There is no venous reservoir comparable to the sinus venosus on the left side of the heart The common pulmonary vein is formed by fusion of the two pulmonary veins returning from each lung The vessel passes alongside the sinus venosus in actual structural contact with the latter The movements of the sinus venosus will possibly result in some movements of the pulmonary vein that may have an indirect propulsive effect on blood in this vessel

Like the right atrium the left has a distinct central lumen which fills directly from the pulmonary vein (Fig 15A) According to Francis (1934) there are no valves at this point It was observed in the present study that a small communication between the pulmonary vein and the left atrium persisted also during atrial contraction but no reflux into the pulmonary vein could be observed The inflow tract however was long and relatively narrow lumened and regurgitation into the pulmonary veins may be prevented by a narrowing of the inflow tract during atrial contraction Zullich (1930) working on *Salamandra maculosa* suggested that the ostium of the pulmonary vein may be closed by the contraction of the adjoining atrial myocardium The central part of the left atrial lumen was circular in shape and the numerous projections leading off from it gave the appearance of a triangular shape when filled maximally (Fig 15B) The left atrial contraction opened the atrio ventricular connection which was far smaller than the connection between the right atrium and the common ventricle (Fig 15A) The ventricle was filled in an upper left position when the left atrium contracted (Fig 15B) The inflow outlined the ventricle in furrows arranged in parallel much like the inflow pattern from the right atrium During the subsequent ventricular ejection this arrangement was retained A detailed description of the selective distribution in the ventricle can be found in Part Six In consonance with conditions on the right side the left atrium also displayed a variable residual volume

Ventricular contraction opened the valves to the bulbus, and the subsequent ventricular ejection filled the arterial trunks The first set of bulbar valves (phylangium valves) can be seen situated quite far up into the bulbus cordis (Fig 16) If contrast was injected in the posterior

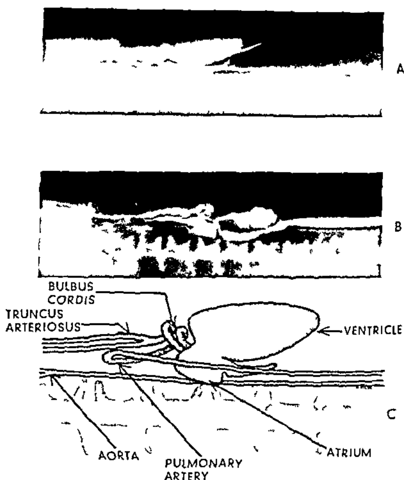


FIGURE SEVENTEEN

Demonstration of the outflow pattern from the contracting ventricle recorded in side projection with horizontal direction of the roentgen beam There is no significant contrast sedimentation A Contrast injection in the pulmonary vein B Contrast injection in the posterior vena cava Exposures at 50 Kv Exposure time 0 03 seconds C Schematical drawing of the outflow pattern from the ventricle

It seems of significance that ventricular systole is timed with the filling phase of the sinus venosus (Fig 14) Bohme (1936), using radiological methods has explicitly demonstrated that ventricular systole facilitates atrial filling in mammals Brünings (1899) made similar claims for fish hearts Benninghoff (1935) compared amphibian larvae and embryos of fishes and birds He concluded that ventricular systole exerted a sucking attraction on blood in the greater veins

The atria (or more specifically in the present context the right atrium) are very important chambers of the heart in *Amphiuma* The right atrium is more than three times larger than the left and can accommodate a volume far exceeding the end diastolic volume of the common ventricle The numerous fingerlike cavities projecting from the walls of the atria undoubtedly contribute to the large capacity factor The fact that the filling of these diverticula was related to the volume inflow suggests that they represent a reserve atrial space which at times of resting heart activity does not contribute to the current of blood passing through the heart but is rather more of a dead space filled with stagnant blood It is conceivable that an increased demand on the heart will increase the vigor of atrial contraction and empty the diverticula Their significance may be thought of as a functional reserve, not only for accommodation of a larger volume but also to facilitate an increased contractility In this regard it is noteworthy that the reserve space is composed of numerous small cylinders which by their contraction can create a higher pressure with less tension needed on account of their small radius compared to a cylinder or main chamber of larger dimensions

The greatest difference in atrial preformance compared with conditions in a mammal is the role of the atrium in ventricular filling In *Amphiuma* ventricular filling is solely the result of atrial contraction The atrio ventricular valves are open for a comparatively short period during which a very rapid atrial contraction takes place The effectiveness of right atrial contraction for ventricular filling depends on the presence of effective valves between the right atrium and the sinus venosus Comparable valves are not present in the mammalian heart In mammals however the contribution of atrial contraction to ventricular filling seem to vary greatly A rapid phase of ventricular filling generally terminates early in diastole The blood responsible for this filling flows in from the great veins through the right atrium The period of rapid ventricular inflow is followed by a quick transition to a period of slower filling which finally transcends into atrial systole Wiggers and Katz (1922) estimated that atrial contraction accounts for 18 60 per cent of ventricular filling Lind, Wegelius and Lichtenstein (1954) demonstrated that the atria of mammals empty more completely if they contract very

vena cava and passed on to the right portion of the ventricle, this selective distribution of blood persisted and blood passed on selectively to the pulmonary arterial trunks The ventricular residual volume varied greatly and was presumably related to the end diastolic ventricular volume and the force of the subsequent contraction The ventricular discharge followed discrete paths from the ventricle up through the incompletely divided bulbus and on to the various arterial conduits This highly selective pattern was obviously related to a strictly laminar outflow which in turn depended on the typical filling in discrete furrows and the retention of this distribution during the subsequent ventricular contraction Figure 17 A, B, demonstrates the laminar outflow pattern recorded *in side projection with contrast injected into the* pulmonary vein There was no significant sedimentation of the contrast medium

DISCUSSION

Figure 14 summarizes the timing and sequential coordination of the various cardiac chambers in *Amphiuma* The interrelationships of these and their role in the propulsing activity of the heart are significantly at variance with conditions in higher vertebrates, notably mammals Davies and Francis (1941) have described the course of the wave of contraction in the salamander heart, *Salamandra salamandra*, by cine photographic technique Their experimental procedure and instrumental technique as well as the size and rate of contraction of the heart are at great variance with the present conditions However, a comparison reveals correspondence in contraction and relaxation time of the right atrium and the ventricle

The presence of a rhythmic contraction in the sinus venosus makes right atrial filling an active process Moreover the capacity for storage in the sinus venosus provides a source for a cardiac reserve that can easily be mobilized at times of demand for an increased cardiac output

In the vertebrate series the sinus venosus exists in its original form in fishes as a fusion of the two ducts of Cuvier It is the point of origin of the whole contractile process that proceeds to the atrium and later sweeps over the ventricle and bulbus The sinus venosus is retained as a discrete cardiac compartment in all vertebrates below birds In birds and especially in mammals the sinus venosus is markedly reduced and has become incorporated in the right atrium In a cineradiographic study of the snake heart, Johansen and Hol (1960) demonstrated a rhythmic blood propelling activity of the sinus venosus In amphibians the present study provides evidence of the importance of the sinus venosus in cardiac performance

refuted for the turtle by Woodbury and Robertson (1942) Studies on mammalian hearts have substantiated the former view (Rushmer 1955)

Quite often one could observe in the studies on *Amphiuma* that a large heart responded with rather weak contractions That this phenomenon did not characterize a failing heart but could be ascribed to changes in the contractile state of the myocardium was demonstrated in several ways The stroke volume often increased spontaneously and ventricular discharge was markedly influenced by administration of cathecholamines such as nor epinephrine (Parts Two and Four)

The cinefluorographic films disclosed rather marked changes in the dimensions of the actively contracting bulbus cordis during the cardiac cycle An accurate analysis of these movements however was hampered by the fact that the atria occupied the same area on the films The hemodynamics of the bulbus cordis is discussed separately in Part Four

SUMMARY

Angiocardiography with cinefluorography and high speed film changer technique have been used to study functional aspects of the cardiac cycle in *Amphiuma tridactylum*

The propagation of contrast from the posterior vena cava or the pulmonary vein through the heart is described The sinus venosus contraction is responsible for a significant part of atrial filling The right atrium is larger than the left The atria form an important capacity space and their contraction is responsible for the entire process of ventricular filling Ventricular filling is short lasting whereas the ventricular contraction and ejection last long compared to conditions in higher vertebrates The residual volumes of all the cardiac chambers vary greatly

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early in diastole than if they contract after the ventricle is distended Cannon *et al* (1950), doing cinefluorographic angiocardiography of dogs, observed a positive correlation between the reduction in atrial dimensions and the stroke change in the projected ventricular area This latter point suggests that the contribution of atrial contraction to ventricular filling is significant enough to influence the cardiac output also in mammals This statement brings up the key role of atrial contraction in cardiac output in *Amphiuma* In *Amphiuma* the end diastolic ventricular volume and thus the degree of ventricular distension are determined solely by the output of the atrium, except for possible changes in distensibility of the ventricle This puts direct emphasis on the atrial contraction as one determinant of the cardiac output The author believes that the larger role of atrial function in cardiac performance demonstrated so clearly in *Amphiuma*, is a character common to all the lower vertebrates It has been demonstrated in the lowest vertebrates (the cyclostomes) by Hol and Johansen (1960) In teleosts the atrium is similarly a sole contributor to ventricular filling (Johansen unpublished) Working with reptiles (snakes) Johansen (1959) and Johansen and Hol (1960) claimed that ventricular filling chiefly takes place during atrial contraction and thereby differs from ventricular filling in mammals, which mainly takes place prior to and during ventricular diastasis

Ventricular contraction and the accompanying ejection last for a comparatively long time in *Amphiuma* (Fig 14) They occupy twice the time for ventricular filling If we set the time lapse for ventricular filling in *Amphiuma* at one, then the volume changes during ventricular contraction last twice as long In comparison a filling time set at one for a mammal would be followed by a contraction giving volume changes lasting only one third of that time The ejection pattern varied greatly In the experiment supporting Figure 14 the ejection proceeded at a fairly steady rate all through the ejection phase In other cases there was commonly a rapid initial phase of ejection declining gradually throughout systole

The roentgen films as well as direct visual observation demonstrated that an increase in the ventricular discharge could also result from a more complete ventricular ejection rather than by a great diastolic filling In the radiological studies this fact was verified from the great variance in ventricular residual volume The sinus venosus and atria behaved in a similar fashion, and it is beyond doubt that the residual volume in all chambers is an important means for temporary increase in cardiac output A similar reasoning seems to apply to reptilian hearts (Johansen and Hol 1960) and to the amphibian anuran heart (Shannon and Wiggers 1939, see also detailed discussion in Part Five), although

PART FOUR

The functioning of the bulbus cordis in *Amphiuma tridactylum*

The myocardial segment designated as the bulbus cordis appears as a developmental feature in all vertebrate embryos. In the course of vertebrate evolution there has been a gradual transformation of the bulbus cordis segment. In elasmobranchs, ganoid and dipnoan fishes and amphibians it exists as a distinct cardiac compartment. In teleosts it is reduced but the nonhomologous bulbus arteriosus composed of smooth muscle and elastic tissue exerts a similar function (Mott 1950, Johansen 1962). In reptiles the bulbus cordis represents a discrete segment at the base of the ventricle with its distal portion absorbed into the truncus arteriosus (Greil 1903). With the development of an anatomically complete double circulation the proximal bulbar ridges participate in the completion of the intraventricular septum and the proximal bulbus is incorporated into the developing ventricle and forms the base of the pulmonary outflow tract in the right ventricular infundibulum (March 1961). Functional importance was early attached to the bulbus cordis. Muller (1839) designated the bulbus as an important accessory heart, and Brucke (1852) ascribed to the bulbus segment a role as an elastic regulator of the pressure impetus on the gills in fishes.

Brucke's (1852) idea of the bulbus as a safeguard against damage to the delicate respiratory capillaries has been supported and elaborated on by several workers. Thus Keith (1924) wrote in a paper on the fate of the bulbus cordis: 'No doubt between the strokes of the ventricular pump the musculature of the bulbus maintains a steady flow of blood but its main purpose seems to be that of a shock absorber — a safety mechanism which prevents the ventricular pump in states of extreme exertion from damaging the re p r... ory network of vessels.' The present investigation aims at clarifying how the bulbus cordis forms a functional link between the discharging ventricle and the receiving arterial tree in *Amphiuma tridactylum*.

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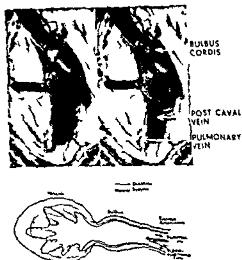


FIGURE EIGHTEEN

Demonstration of the dimensional changes of the bulbus cordis during a cardiac cycle A Ventricular diastole B Late ventricular systole Note the increase in the ventricular outflow tract A and B seen in ventral aspect C Schematic drawing of the dimensional changes during ventricular contraction

The direct confluence of the bulbus cordis with the ventricular myocardium is immediately suggestive of an active role in the cardiac cycle By visual inspection or from cinefluorographic films of a beating heart one can clearly follow the movements of the various parts During the early part of ventricular systole the bulbus cordis is swiftly distended by the outflowing blood Later the bulbus cordis contracts and displays a marked reduction in its diameter Figure 18 illustrates this point There is a manifest widening of the orifice of the ventricular outflow tract during its most active ejection phase (Fig 18B) The increase could amount to 20% compared to the smallest value during the subsequent bulbar contraction (Fig 18C) The largest dimensional changes of the bulbus cordis took place about midway between the base of the ventricle and the truncus arteriosus At this level a doubling of the diameter was apparent during vigorous ventricular contractions This means that the volume changes the bulbus cordis undergoes may be of the order of at least one to four

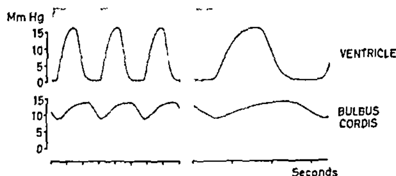


FIGURE NINETEEN
Simultaneous recordings of pressure from the ventricle (top tracing) and the bulbus cordis Time marks one second

Figure 19 demonstrates simultaneous records of pressure recorded from the ventricle (top tracing) and the bulbus cordis less than 1 cm from the ventricle There is a conspicuous difference in the slope of the two tracings The rate of change of pressure (dp/dt) was more than seven times greater for the ventricle than for the bulbus in their ascending pressure slopes Note also that the peak pressure in the bulbus cordis appeared far later than peak intraventricular pressure *In fact it was* reached at a time when the intraventricular pressure had fallen to its *minimum* value

In most cases the highly compliant, elastic walls of the bulbus cordis made it impossible to distinguish the phase of distension from the subsequent phase of contraction on the pressure records In some cases,

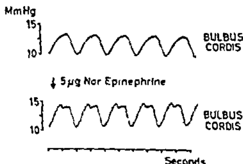


FIGURE TWENTY
Pressure records from the bulbus cordis before (upper tracing) and after injection of 5 μg nor-epinephrine Note the steeper pressure slope and the biphasic peak in the bottom record Time marks one second

however particularly at very forceful heart beats, a distinct double pulse was discernible Figure 20 depicts two pressure records recorded from the same animal The bottom tracing was recorded subsequent to an injection of 5μg nor epinephrine As is readily apparent nor epinephrine affected the contractility or the rate of change in shortening the contracting ventricle This became evident through a markedly increased rate of change of pressure (dp/dt) although the increase in the general level of pressure was moderate The more vigorous contraction became manifest as a double pulse in the bulbus cordis pressure record in which the second pulse can be referred to the contraction of the bulbus itself The distension contraction sequence of the bulbus cordis could similarly become discernible as a marked change in the rising pressure slope



FIGURE TWENTY-ONE
Simultaneous recordings of pressure from the bulbus cordis (lower tracing) and the ventricle
Time marks one second

without a distinct double pulse being present Figure 21 illustrates this point The initial rapid pressure rise stemmed from the inflow of the ventricular discharge while the subsequent more gentle pressure rise occurred as a result of the contraction of the bulbus itself

Figure 22 demonstrates how the intraventricular pressure gives rise to an arterial pulse wave and how the propagation of this is related to the characteristics of the bulbus cordis The depulsation in the bulbus occurred at the obvious expense of the level of systolic pressure produced by the ventricle The peripheral pulse wave however showed a gentle diastolic run off which must be of special value for a continued circulation at such low heart rates

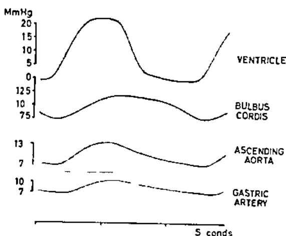


FIGURE TWENTY TWO
Simultaneous recordings of pressure from the ventricle (top tracing) the bulbus cordis the ascending aorta and a branch of the gastric artery Time marks one second

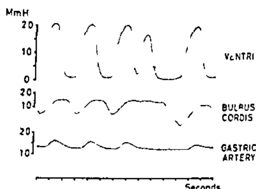


FIGURE TWENTY THREE
Simultaneous recordings of pressure from the ventricle (top tracing) the bulbus cordis and a gastric artery branch The extra systole demonstrates the pressure chamber effect exerted by the bulbus cordis
Time marks one second

Figure 23 is particularly illuminating in its demonstration of the pressure chamber effect exerted by the bulbus cordis A spontaneous ventricular extra systole occurred shortly after the third heart beat, which was then followed by a compensatory pause The extra systolic pressure peak is hardly recognizable in the bulbus cordis pressure record, which demonstrates how the energy released by the extra systole was stored as potential energy in the bulbus The bulbus cordis finally relaxed toward the end of the compensatory pause and released its potential energy to the peripheral arteries The net effect was that the extra systole was not perceptible in the peripheral arterial pressure, which was kept at a steady diastolic level during the entire course of the extra systole

One would predict a greater importance of the bulbar pressure chamber mechanism at the lower heart rates Figure 24 attempts to illustrate this point The first portion of the record was recorded while the heart was under the influence of epinephrine, while the last part was recorded subsequent to intravenous administration of 4 µg acetyl choline, which gave rise to a marked bradycardia Note the conspicuous difference in the appearance of the bulbus cordis pressure record in the two situations During tachycardia the pressure slopes were not much at variance with the slope of the intraventricular pressure The bulbus contraction extended the pressure into ventricular diastole but did not add to the pres

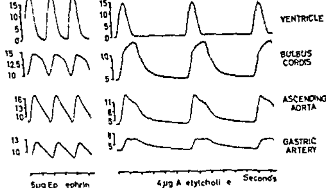


FIGURE TWENTY FOUR

Simultaneous recordings of pressure from the ventricle (top tracing) the bulbus cordis the ascending aorta and a branch of the gastric artery The tracings to the left were recorded while the heart was under influence of epinephrine while the bradycardia to the right resulted from injection of acetyl choline Note the marked difference in th appearance of the bulbus cordis pressure record Time marks one second

sure level created by ventricular contraction During the pronounced bradycardia however the bulbus contraction changed the appearance of the pre sure pulse markedly and added to the general level of pressure More peripherally the bulbu effect became manifest as a biphasic wave in the pressure records from the aorta and the gastric artery, and resulted in a maintenance of systolic pressure for an extended time in the peripheral arteries An extraordinarily gentle diastolic run off is seen to keep the diastolic pressure up in spite of the profound bradycardia

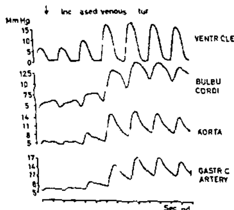


FIGURE TWENTY FIVE

Simultaneous recordings of pressure from the ventricle (top tracing) the bulbus cordis the aorta and a branch of the gastric artery during a period of severely weak heart action followed by an increased venous return Time mark one s cond

Figure 25 depicts simultaneous pressure recordings from the ventricle the bulbus cordis the ascending part of the right aorta and from a branch

of a gastric artery The first part of the recordings were made during an extremely weak heart action The stroke volume discharged from the ventricle at this time was evidently too small to give rise to any significant pressure rise in the bulbus It is noteworthy, however, that the bulbus cordis contraction (first two heart beats) appears as a distinct second pulse and adds to the general level of systolic pressure At the time indicated by the arrow an increase in venous return was applied by infusion of Ringer solution into a mesenteric vein Within a very short time the ventricular pressure was more than doubled The bulbus cordis pressure similarly underwent a marked increase as well as a change in general appearance As the force of contraction increased, the rate of change of pressure (dp/dt), and the pulse pressure increased sharply, and already the subsequent heart beat resulted in a threefold increase in pressure peripherally and a doubling of the pulse pressure The rounded top of the bulbus cordis pressure record demonstrates the pressure chamber effect The rapidly increasing levels of diastolic pressure reached peripherally disclose the key role of the bulbus as an energy distributor

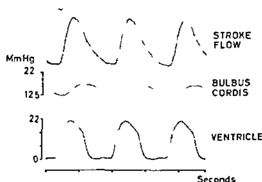


FIGURE TWENTY SIX
Simultaneous recordings of stroke flow and pressures in the bulbus cordis and the ventricle Note the depulsating effect of the bulbus cordis and the maintenance of positive flow through the entire cardiac cycle Time marks one second

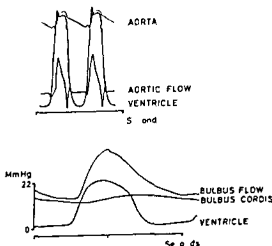


FIGURE TWENTY SEVEN
Contours and time relationships of intraventricular and aortic pressure together with pulsatile outflow from the heart A shows conditions in a dog (redrawn from Spencer and Greiss 1962) B shows the results obtained presently on *Amphiuma* Note the marked depulsating effect of the bulbus cordis and the maintenance of flow throughout the cardiac cycle

Figures 26 and 27 throw light upon the importance of the bulbus cordis in the adjustment of the outflow from the heart The upper set of records Fig 27 (A), is redrawn from Spencer and Greiss (1962), and shows contours and time relationships of aortic pressure left ventricular pressure and the flow in the ascending aorta of a dog In Fig 27 B are shown the corresponding parameters recorded in the present study on *Amphiuma* Note the marked depulsating effect of the bulbus cordis and the maintenance of positive flow through the entire cardiac cycle

DISCUSSION

The role of the larger arteries in exerting a pressure chamber effect, or Windkessel effect has been amply evaluated in regard to the mammal This function in brief provides the larger arteries with a rhythmic, highly pulsatile input from the heart and then converts this to a smooth output to the peripheral arteries

In the lower vertebrates the difference in anatomical structure as well as a more slow heart action focus the emphasis on the bulbus cordis as the important site for pressure chamber activity A low frequency, low pressure vascular system must have means to distribute the energy released from ventricular contraction over an extended time if a continuous circulation is to be achieved

The large variations measured in the dimensions of the bulbus cordis associated with the cycles of distension and contraction have several consequences An increase in the ventricular outflow orifice during the most vigorous ejection phase (Fig 18) will favor an increased output by reducing the outflow resistance

To be able to receive the ventricular discharge and force it onwards, the bulbus cordis is endowed with an exceptional elasticity Valves situated within this highly compliant structure would not be mechanically competent were they not furnished with a muscular support such as that provided by the bulbus This adds to the importance of the bulbus cordis in the lower vertebrates as a support for and a supplement to the action of the ventricular semilunar outflow valves (Keith 1904 1924 March 1961) Keith (1904) makes the interesting point that the lower vertebrate bulbus segment divides during phylogeny to form the roots of the aorta and pulmonary artery but while its musculature disappears from the aorta it remains as loop which encircle the terminal part of the infundibulum of the right ventricle and take their fixed origin from the base of the aorta In all vertebrates the efficiency of the semilunar valves depends on this musculature

As early as 1834 Weber postulated that the elastic properties and the

distensibility of the arterial tree enabled it to receive the ejected stroke volume, and that subsequently the stretched arterial walls provided the propulsive force which caused blood to flow not only in systole but also during diastole The present use of the term 'Windkessel effect' for this function derives from Weber He compared the elastic storage capacity of the arterial tree with physical 'Windkessel' devices such as the manually operated fire engines or the compressed air reservoirs of pipe organs Weber reasoned that ejection into an elastic outflow system would save work because pulsating flow in a rigid outflow system would require acceleration of the stroke volume as well as of the large arterial blood column at the beginning of systole

The idea that a rational relationship exists between cardiac function and the elastic storage capacity of the outflow system takes on importance when considered in a *phylogenetical context In the original one* circuit vascular system of fishes the heart is primarily a gill pump where the remaining pressure head, after traversing the respiratory capillary *bed, represents the propulsive force for the systemic circulation A respira*tory network of vessels in the immediate proximity of the main pump seems in obvious need of mechanisms to protect the delicate vessels against sudden changes in pressure The bulbus segment interposed between the heart and the gills performs a crucial task in this respect by providing a depulsating effect on the ventricular pressure pulses Radiological studies on the eel (Mott 1950) demonstrated an exceptional elasticity of the bulbus arteriosus tissue From pressure and flow measurements a conspicuous depulsating effect of the bulbus arteriosus in the cod (*Gadus morhua*) has been demonstrated (Johansen 1962) The centraliza*tion of the important depulsating function in the* bulbus arteriosus segment of fishes is retained in the bulbus cordis of amphibians and partly in the reptiles toward the development of a complete double circulation in *birds and mammals In birds and mammals* the discrete, effective, depulsating activity of the bulbus is lost and the 'Windkessel function resides in the entire system of the larger arteries An interesting comparison between *conditions in lower and higher vertebrates*, however, can again be made The elasticity of the higher vertebrate aorta has a definite limit at which further expansibility is halted This limit differs very much from *conditions in the pulmonary circuit* where pressure increments several times the original pressure may be tolerated before the same limit is reached The connection of the bulbus in the lower vertebrates with a *low pressure, low frequency system* shows in this respect a parallel to the low pressure system of the higher vertebrate pulmonary circuit which has descended anatomically from the same segment

The data presently obtained provide experimental proof of a number of suppositions advanced to explain the role of the bulbus cordis Figures 19 22 and 23 demonstrate clearly not only the marked depulsating effect but also the fact that bulbus contraction persists into ventricular diastole thereby affording significant support for the bulbar valves

The changes in the sequence of distension and contraction of the bulbus segment at weak heart actions (Fig 25), or in response to epinephrine and acetylcholine (Figs 20, 24), are difficult to interpret in particular because the components of the ventricular and bulbar contractions are not always distinguishable in the pressure record At times of severely weak *ventricular contractions* (*Fig* 25), *when the bulbar distension from* the ventricular discharge was reduced or abolished there was a noticeably persistent phase of bulbar contraction The data furthermore, justify the assumption that rapid and pronounced bulbar distension during vigorous ventricular contraction is followed by a subsequent forceful bulbar contraction In this regard the time course of the initial bulbar pressure rise seems to have a decisive effect (Fig 20)

A number of authors have emphasized a rational relationship between cardiac performance and the elastic properties of the outflow system (Knebel 1941 a, b Alexander 1952, Kenner 1959) With particular reference to the arterial pressure levels the form of the pressure pulses and how these are influenced by outflow elasticity, Wegler and Boger (1939) and Wiggers (1938) have added important information

Salisbury *et al* (1962) have investigated the influence on cardiac function exerted by the elasticity of the outflow system under rigidly controlled experimental conditions At slow heart *rates the diastolic* pressure in rigid outflow systems reflected the level of controlled resistance When more distensible systems were interposed between the ventricle and the controllable arterial reservoir at the same level of resistance, higher diastolic pressures were observed, which they *interpret* to be a *function* of the elastic force applied in diastole to the blood column by the distensible outflow system

In general lower vertebrates have low heart rates and the *pressure* chamber effect in the bulbus cordis is probably a *significant* contributing *factor to* the maintenance of adequate diastolic pressures The data acquired in the present study give ample experimental *support to this* supposition (Figs 23 24 and 25) Figures 19 21 and 26 all demonstrate that the rapidly developing pressure in the ventricle during contraction is converted in the bulbus to a *smooth long* lasting pressure output It is apparent from Figure 19 that the maximal pressure reached in the bulbus is far lower than maximal intraventricular pressure There is thus a positive pressure gradient from ventricle *to* bulbus during the entire

period of ventricular outflow Recent studies on mammals (Spencer and Greiss 1962) have shown that there is only a very short period during which outflow from the left ventricle follows a positive pressure gradient The major outflow takes place against a pressure gradient propelled by the inertia of the initial impact of ventricular pressure (Fig 2/A) Available evidence suggests that the right ventricular ejection follows a positive pressure gradient all through the ejection phase also in mammals (Johansen, unpublished) The significance of this difference is amplified when remembering that the bulbus segment in the mammal remains as loops which encircle the terminal part of the infundibulum The large pressure gradient from ventricle to bulbus in *Amphiuma* may result in a high velocity of the blood on its passage from the ventricle to the first part of the bulbus Such a high initial velocity may be related to the presence of a typical laminar outflow pattern, which in turn will be of significance for the mixing of systemic and pulmonary blood (Part Six)

The alleged role of the bulbus in maintaining aortic flow in late systole has hitherto not been experimentally proven The present results (Figs 26 and 27) demonstrate that positive flow in *Amphiuma* lasts during the entire cardiac cycle, thereby adding another conspicuous difference between the higher and lower vertebrates Spencer and Greiss (1962) have demonstrated that positive aortic flow prevails in less than one third of the cardiac cycle in mammals (dogs) Figure 27 compares the results of Spencer and Greiss (1962) (top figure) with those of the present investigation In *Amphiuma*, with a heart rate almost 1/4 of that in the dog, the extreme depulsating effect of the bulbus helps maintain a positive flow throughout the entire cardiac cycle After the short period of positive aortic flow on the dog there follows an abrupt shortlasting period of reversed flow, which terminates in a phase of zero flow The significance of a maintained flow as demonstrated in *Amphiuma* is enhanced when the normally prevailing low heart rate is considered March (1962) studied the movements of the bulbus cordis segment in elasmobranchs with cinematographic technique He concluded that the bulbus cordis exerts its influence by limiting the ventral aortic pressure variations in early systole when a rapid flow through the gills can be achieved by a small trans gill pressure gradient The active contraction of the bulbus in late systole provides an additional discharge which maintains pressure and flow through the gills in the period of highest pressure in the dorsal aorta As a consequence he thought that gill flow is kept up until closure of the semilunar valves Satchell (1960), also studying elasmobranchs, took differential pressure measurements and evaluated a lasting period of flow on this basis It remained to take direct measurements of flow in the

ventral aorta Such studies have been done recently on the teleost, *Gadus morhua* (Johansen 1962) A conspicuous pressure chamber effect of the bulbus arteriosus and a long lasting aortic outflow were demonstrated

SUMMARY

The functional importance of the highly elastic and muscular bulbus cordis segment of the amphibian heart has been evaluated from measurements of intracardiac and intravascular pressures taken while monitoring flow in the bulbus cordis The main role of the bulbus cordis is its marked pressure chamber function The depulsating effect tends to keep up the diastolic levels of pressure in the peripheral arteries at low heart rates Another consequence is the distributing effect on the ventricular discharge which results in a maintained positive outflow from the heart throughout the cardiac cycle even at very low heart rates The bulbus cordis is also important in assisting the valve function and preventing regurgitation of the ventricular outflow

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PART FIVE

Cardiac output and dynamics of cardiac performance in *Amphiuma tridactylum*

The functional interrelationships between the various cardiac compartments and the larger conduits connected to the heart in the lower vertebrates is a subject of which very little is known

No measurements of intracardiac and intravascular pressures with simultaneous recording of stroke volume and cardiac output have been reported for any amphibian

The experiments to be described are attempts to analyze cardiac performance in *Amphiuma tridactylum* based on simultaneous measurements of pressure and flow using modern cardiovascular instruments

EVALUATION OF THE CARDIAC CYCLE FROM MEASUREMENT OF INTRACARDIAC PRESSURES

Results and Comments

The heart of *Amphiuma* consists of five distinctly separate parts all of which contribute to the propulsion of blood by active contractions These are the sinus venosus the two atria the common ventricle and the bulbus cordis At room temperature (18 20) the heart rate in a quietly resting or lightly anesthetized specimen is around 25 beats per minute

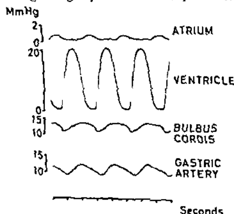


FIGURE TWENTY EIGHT

Simultaneous pressure recordings from the right atrium the ventricle the bulbus cordis and a branch of the gastric artery demonstrating the successive contractions of the various chambers in the heart of *Amphiuma tridactylum* Time marks one second

Figure 28 demonstrates the successive contractions of the various chambers in the heart of *Amphiuma*. The first mechanical event of a cardiac cycle was a slow pressure rise in the sinus venosus. This contraction proceeded much in the manner of a peristaltic wave as well as a general shortening of the sinus venosus segment, starting at a level corresponding to the apex of the distended ventricle and passing anteriorly toward the right atrium. Both the sinus venosus and the right atrium are typical capacity spaces and only small increments of pressure in the sinus venosus will suffice to propel a sizable volume against a small resistance into the right atrial cavity.

The right atrial pressure curve has a very conspicuous appearance. In some of the records a smaller pressure wave preceded the actual contractions of the atrium (Fig. 28). This small wave most likely represented the contraction wave in the sinus venosus, transmitted into the

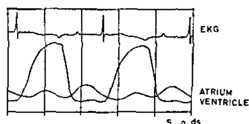


FIGURE TWENTY NINE

Demonstration of the time relations between the components of the electrocardiogram and the atrial and ventricular pressure records. Time marks one second.

atrium through the open sino atrial connection. Figure 29 demonstrates the time relations between the electrocardiogram and the atrial and ventricular pressure records. The P Q interval of the EKG is particularly extended compared to the sequence of excitation in higher vertebrate forms. Accordingly, practically the whole period of ventricular relaxation and diastasis is occupied by atrial contraction. Ventricular excitation and contraction, the Q T interval, occupies a considerable portion of the cardiac cycle. The T P interval is therefore of a very short duration and radically different from conditions in electrocardiograms from avian and mammalian hearts. The same short T P interval has earlier been emphasized by Davies *et al.* (1951) in *Crocodilia*, Johansen (1959) in the snake heart and by Mullen (1962) in two species of iguanid lizards.

Figures 28 and 29 provide substantial support for the major conclusions drawn in the radiological analysis of the cardiac cycle in *Amphiuma* (Part Three). First of all it emphasizes the paramount importance of atrial activity for cardiac performance. Atrial contraction coincides with ventricular relaxation and is thus entirely responsible for ventricular filling. Ventricular contraction is comparatively long lasting, and the period available for filling, is accordingly shorter.

INCREASED LOAD ON THE SINUS VENOSUS AND RIGHT ATRIUM

Results and Comments

It has been pointed out that venous pressure and the return of blood to the heart is markedly influenced by muscular movements in the intact

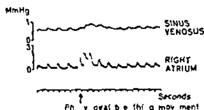


FIGURE THIRTY
Simultaneous pressure recordings from the sinus venosus and right atrium Pharyngeal breathing movements were followed by a rise in pressure and rate of contraction Time marks one second

Amphiuma (Part Two) Figure 30 demonstrates several points of interest in this connection Simultaneous recordings of pressures in the sinus venosus and right atrium were done on a spontaneously breathing specimen The arrow indicates a period of spontaneous pharyngeal breathing movements Such movements were accompanied by increased tension in the general body musculature The breathing movements were followed by a rise in venous pressure which was transmitted to the sinus venosus The sinus venosus pressure rose and, more important, its frequency of contraction nearly doubled These responses are even more pronounced when the atrial pressure curve is considered Thus the systolic atrial pressure increased threefold from one beat to the next The frequency of contraction was similarly doubled The Figure demonstrates how a naturally occurring increase in venous return evokes a prompt increase in both

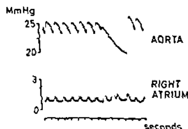


FIGURE THIRTY-ONE
Simultaneous pressure recordings from the aorta and right atrium A short period of ventricular asystole is promptly followed by an increase in the force of contraction of the right atrium Time marks one second

frequency and force of contraction of the receiving chambers the sinus venosus and right atrium Figure 31 demonstrates the prompt response of the atrium[1] to an increase in outflow resistance The ventricle had stopped contracting spontaneously for three beats and ventricular

[1] Unless otherwise stated the atrium referred to is the right

ejection was consequently temporarily halted This in turn represented an increase in resistance to the still beating atria, which immediately increased their force of contraction

These findings demonstrate the significant role played by the sinus venosus and atrium for the filling of the ventricle An increased load on these chambers, whether in the form of an increased venous return or an increased outflow resistance, will produce an immediate augmentation in both rate and force of contraction

THE ROLE OF THE INTRAPERICARDIAL PRESSURE IN THE FILLING OF THE HEART

Results and Comments

Intrapericardial pressure fluctuations set up by the changes in volume of the contracting ventricle, and similar fluctuations related to the breathing movements, have been reported in a number of gill breathing vertebrates (Brunings, 1899, in teleosts and Lyon, 1928, in elasmobranchs) Such pressure changes will in all likelihood transmit through the thin walled atria and hence influence the resistance to atrial inflow In fishes, several investigators have recorded negative pressures in the sinus venosus and greater veins Negative venous pressures were never recorded during the present study Obviously, pressure measurements alone can only contribute indirect evidence but can never conclusively decide whether a *vis a fronte,* or sucking action of the heart, is created by the contraction of the ventricular myocardium However, a facilitation of atrial filling will most likely result at times when intrapericardial pressure falls Even a small *decrease of atrial pressure concurrent with ventricular systole* can be expected to have a great effect on inflow because of the low resistance in the sino atrial inflow tract It was demonstrated in the present study that at each ventricular contraction there was a drop in intrapericardial pressure (Fig 32)

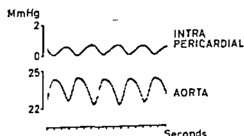


FIGURE THIRTY TWO
Cyclic changes in intrapericardial pressure related to aortic pressure (ventricular contraction) Time marks one second

To elucidate more fully the importance of the intrapericardial pressure for the cardiac performance, attempts were made to produce slight intrapericardial pressure changes when recording the aortic blood

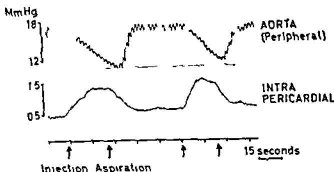


FIGURE THIRTY THREE
Simultaneous recordings of aortic and intrapericardial pressure When the intra pericardial pressure was artificially raised by injection of saline cardiac filling was impeded and aortic pressure fell rapidly Time marks one second

pressure Figure 33 depicts the results of such a procedure At the point of the arrow a small volume of Ringer solution, 0 2 0 4 ml, was slowly introduced into the pericardium This increased the intrapericardial pressure and resulted in a concurrent drop in arterial pressure As soon as the volume added to the pericardium was withdrawn, the intrapericardial pressure dropped and the initial values of arterial pressure were quickly re established From visual inspection of the beating heart it became apparent that the increased intrapericardial pressure first of all impeded the filling of the heart and did not hinder the subsequent contraction It seems of particular interest that only small increases in intrapericardial volume could effect a sizable change in cardiac performance

In mammals the theory of an attraction of venous blood by the contraction of the ventricle has been debated extensively (Brecher 1956) Among a number of workers Hamilton (1930) found it necessary to assume a strong systolic sucking action of the heart upon the *venae cavae* The alleged explanation for the effect in mammals dates back to the finding by Purkinje (1843) that there is an alternating descent and ascent of the atrio-ventricular junction during heart activity The apex of the heart however remains more stationary These movements would result in an enlargement of the atria during ventricular contraction with a consequent sucking action on the blood in the greater veins

No mention except tangentially by Nerlich (1951) seems to have been made in the literature on mammalian physiology of a possible mechanism related to intrapericardial pressure fluctuations Conditions in the lower vertebrates are markedly different however There is no thoracic cavity with a negative pressure Moreover the pericardium is

ejection was consequently temporarily halted This in turn represented an increase in resistance to the still beating atria, which immediately increased their force of contraction

These findings demonstrate the significant role played by the sinus venosus and atrium for the filling of the ventricle An increased load on these chambers, whether in the form of an increased venous return or an increased outflow resistance, will produce an immediate augmentation in both rate and force of contraction

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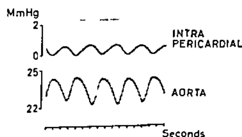


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To elucidate more fully the importance of the intrapericardial pressure for the cardiac performance, attempts were made to produce slight intrapericardial pressure changes when recording the aortic blood

isometric contraction is easily discernible It lasts 0 12 seconds of a cardiac cycle of 1 70 seconds duration at a heart rate of approximately 33 beats per minute The ventricular outflow phase lasts 0 72 seconds Ventricular outflow terminates at the time of closure of the bulbar valves Subsequently ventricular relaxation sets in The period of isometric relaxation lasts about 0 30 seconds and is followed by atrial contraction and renewed ventricular filling The general appearance of the ventricular outflow curve is smooth There is an early rapid ejection phase reaching a summit at peak intraventricular pressure From that point on the velocity of ejection declines gradually, but does not reach zero level until commencement of the next ventricular contraction The flow in the bulbus cordis is always positive and attains zero level only for a brief period just prior to ventricular contraction The gentle decline in outflow velocity during ventricular relaxation is not influenced by closure of the ventricular outflow valves For comparison positive flow in mammals prevails for only about 35% of the cardiac cycle

Cardiac output Its adjustment and regulation

Results

The experiments on cardiac output were all done on immobilized lightly anesthetized animals The measurements were taken with the animal in dorsal position The heart region was carefully exposed the pericardium opened in the midline and retracted bilaterally This gave access to the bulbus cordis and the remainder of the heart and greater vessels Infusion of drugs blood or Ringer solution was done through a catheter in a mesenteric vein Figure 34 demonstrates pulsatile flow in the bulbus together with pressures in the bulbus and the ventricle The bulbus pressure was recorded through a catheter placed somewhat distal to the flow probe The stroke volume in this particular specimen was

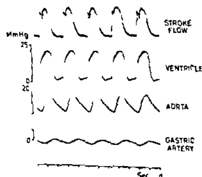


FIGURE THIRTY FIVE
Simultaneous recordings of pulsatile flow in the bulbus (upper tracing) and pressures from the ventricle the aorta and a branch of the gastric artery Note the biphasic flow pattern Time marks one second

never spaced freely, but is generally attached to the heart as well as to adjoining muscular tissue, a condition necessary if a sucking action on the thin walled atria is to exist

In *Amphiuma* it seems reasonable to assume that venous return depends upon the concerted action of both a *vis a tergo* and a *vis a fronte*

Dynamics of ventricular ejection

Results and Comments

In general physiology the traditional concept is taught that ventricular ejection can be pictured as a sawtooth wave with peak velocity occurring early in systole Following the rapid ejection phase the velocity of forward flow decreases gradually until closure of the aortic valves (Spencer and Greiss 1962) Subsequently there is a sharp backflow in the aorta corresponding to the incisura on the pressure pulse During diastole there is zero flow The above description refers to experiments on the left ventricle of mammalian forms Reference of a similar nature to lower vertebrate forms is entirely lacking In the present experiments ventricular ejection in *Amphiuma* was studied by measuring intraventricular and intrabulbar pressures directly These pressures were fed into a differential pressure transducer giving instantaneous values of the existing pressure gradients Stroke flow was monitored simultaneously, using electromagnetic flowmeter technique The flow probe was placed around the distal part of the bulbus cordis Figure 34 depicts the results A period of

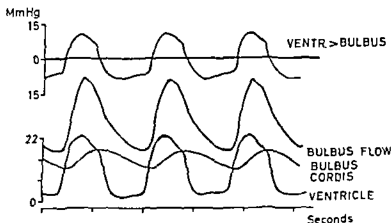


FIGURE THIRTY FOUR

Simultaneous recordings of ventricular outflow measured in the distal part of the bulbus cordis and pressure recordings from the bulbus cordis and ventricle The pressures were fed into a differential pressure transducer giving instantaneous values of the pressure gradient from ventricle to bulbus (upper tracing)

Time marks one second

very quickly to an increased venous return Within a few beats (4 in number) the stroke volume was augmented twofold The ventricular pressure increased by about 14 cm H_2O and the bulbus pressure by about 10 cm H O during the same period The cardiac output increased from 12 95 ml/min to 25 90 ml/min The elevated cardiac output persisted for more than 30 seconds after the actual cessation of infusion This indicates that the initial increase in cardiac output has mobilized the blood reserves and facilitated the force of contraction of the sinus venosus and the atria as well as of the ventricle itself Note however, that the heart rate stayed unchanged during this process

In other experiments a marked tachycardia was observed *in response* to an increased venous return In Figure 37 the heart rate increased from

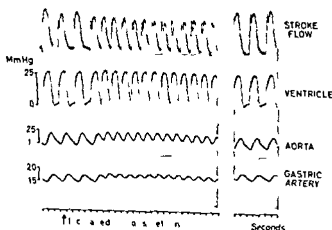


FIGURE THIRTY SEVEN

An artificial increase in venous return evoked a prompt tachycardia The stroke volume fell and cardiac output increased insignificantly Time marks one second

ca 25 to 35 beats per min Within two beats the stroke volume was reduced from 0 45 ml before onset of increased venous backflow to 0 35 ml during the tachycardia The accompanying changes in cardiac output were 11 25 ml/min before and 12 25 ml/min during the infusion Forty seconds later the heart rate was back to the initial level The stroke volume however, was somewhat increased from before onset of venous return being 0 52 ml The intraventricular as well as the intra arterial pressures increased only slightly in response to the increased backflow stimulus The increase in cardiac output was very small compared to the response depicted in Figure 36 in which the heart rate did not change at all In another experiment repeated increases in venous return gave

0 67 *ml* of blood, giving a cardiac output of approximately 22 ml/min The animal weighed 750 g, giving a flow of about 30 ml/kg/min

Figure 35 demonstrates stroke flow and ventricular and arterial pressures Note the sharp notch and subsequent rounded wave in the pulsatile flow record The idea emerged early that the second wave in the biphasic flow curve represented a discharging function of the bulbus cordis The aforementioned dimensional changes of the bulbus may represent a source of error in this regard, as these changes might have influenced the pick up signal of the flow probe However, even with the flow probe in the anterior-most position at the bulbus, where the move ments were minor, the double wave often appeared distinctly The evidence thus supports the view that the initial sharp drop in the flow record can be referred to the distending phase of the elastic bulbus tissue The accompanying rapid volume changes momentarily decelerate the ventricular discharge Subsequently, when the bulbus is maximally dilated and starts contracting, the flow swiftly accelerates again It seems conceivable that the size of the area under this second phase of increased flow will depend upon the extent and force of bulbar contraction, which therefore may provide a real contribution to the discharge of blood from the heart

RESPONSE TO AN INCREASED VENOUS RETURN TO THE HEART

Figure 36 shows a continuous record of pulsatile stroke flow, and bulbus and ventricular pressures during a period of slow infusion of Ringer solu tion into a peripheral vein One can appreciate that the heart responded

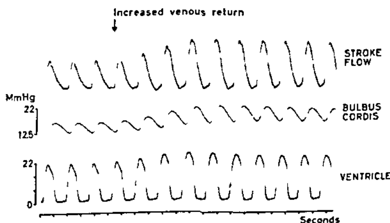


FIGURE THIRTY SIX

Pulsatile ventricular outflow and pressures in the bulbus cordis and ventricle during an artificial increase in venous return The stroke volume increased rapidly giving a doubling of the cardiac output Time marks one second

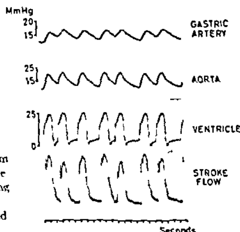


FIGURE THIRTY NINE

Simultaneous records of flow (bottom record) and pressures in the ventricle aorta and a gastric artery branch during a period of arrhythmia

Time marks one second

second beat of the pairs was markedly smaller than in the first beat The reduction could be from 0 84 ml to 0 48 ml in the next contraction Al though the heart at this time worked at large residual volumes in both atria and ventricle this reserve was evidently not mobilized in the in stantaneous regulation of stroke volume However, if the load on the heart was slightly influenced by for instance a lifting of the tail end and thereby an increase in the general arterial pres ure and resistance to the outflowing blood from the heart a sizable increase and consistency in the stroke volume could result The stroke volume could increase up to 50% in a single beat a plain indication of residual blood ready for instant mobilization

In the experiment underlying Figure 40 a small dose of acetylcholine

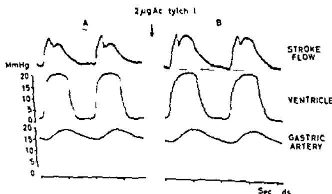


FIGURE FORTY

Response in stroke flow ventricular and arterial pressures to injection of 2 μg acetyl choline giving rise to a sl ght bradycardia The stroke volume increased from 0 64 ml to 1 00 ml The intraventricular pressure increased insignificantly

Time marks one second

examples of the two types of responses described above, *but now occurring* in one and the same animal only a few minutes apart

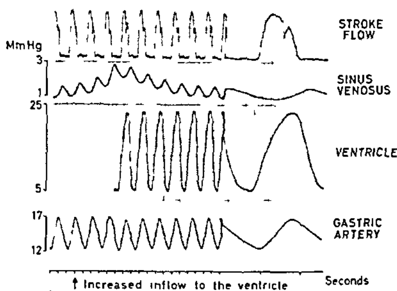


FIGURE THIRTY EIGHT

Simultaneous records of pulsatile outflow from the heart and pressures in the sinus venosus the ventricle and a branch of the gastric artery An increased filling of the ventricle by direct infusion through the cardiac wall gave no response in stroke volume *or frequency of the heart The sinus venosus pressure rose simultaneously without* eliciting any immediate response from the heart Time marks one second

Figure 38 *contains records of outflow from the heart as well as* pressures in the sinus venosus, the ventricle, and a gastric artery branch An increased inflow and distension of the ventricle was effected by direct *infusion of Ringer solution through a needle catheter inserted through the* wall of the ventricle No tachycardia was detectable, neither did the stroke volume increase Meanwhile, the backflow from the greater veins dammed up, resulting in a pressure increase in the sinus venosus The increased pressure did not evoke any responses from the heart During this experiment the heart was working at a large size with a fairly high stroke volume of 0 74 ml The stimulus of an increased backflow was thus certainly not always adequate in itself to bring about an increased cardiac output or a change in rhythm If the stroke volume was fairly high initially, with all the cardiac chambers working at large sizes with *significant residual volumes, the heart generally did not respond* immediately to an increased backflow stimulus

Figure 39 shows a series of records observed during a period of arrhythmia The arrhythmia *consisted in a grouping of the beats in pairs* followed by a compensatory period of rest The stroke volume in the

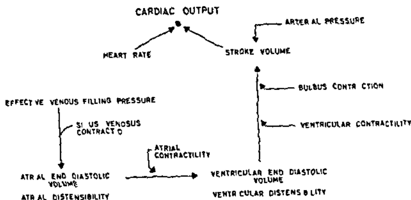


FIGURE FORTY TWO
Simplified scheme of the various factors influencing the cardiac output in *Amphiuma tridactylum*

The available data seem to justify a listing of the following factors as important determinants of cardiac output in *Amphiuma* (Fig 42)

A THE EFFECTIVE FILLING PRESSURE IN THE GREATER VEINS

The pressure gradient toward the heart in the greater veins i e the effective filling pressure seems to be related to the following dynamic factors in *Amphiuma*

1) Force of ventricular ejection with accompanying changes in intra pericardial pressure a *vis a fronte* effect

2) Muscle pumping and tissue pres ure changes the level of which is largely determined by motor activity

3) Influence from the breathing movements Such movements are in general accompanied by a dual effect, one of increased muscle tension related to the pharyngeal breathing movements and the other a direct compressing effect on the visceral veins by the inflated lungs

4) Venous tone Indirect evidence from the response in cardiac output to an artificially applied increase in venous return suggests that changes in venous tone will affect the venous capacity factor and hence influence venous return Similarly experiments with drug administration (epinephrine see Part Two) point toward a reduction in the capacity spaces in all likelihood elicited by changes in venous tone

B SINUS VENOSUS CONTRACTIONS

The sinus venosus exists as a separate cardiac chamber in all lower vertebrates up to and including the reptilia Evidence of active rhythmic contractions of a propulsive nature in the sinus venosus has been presented

resulted in a very slight reduction in heart rate, an increased filling time, and hence a greater distension of the ventricle The stroke volume increased from 0 64 ml in part A to 1 00 ml in part B of the figure The increased filling time as well as changes in myocardial distensibility and amounts of residual blood may be involved in the response obtained

Several investigators have recently emphasized that major factors in the cardiac adjustment may stem from neural and humoral mechanisms which act to alter the inherent distensibility and contractility of the myocardium This view is disregarded in the Starling concept, and has come to play a major part in the revision of the views on cardiac control that have emerged during recent years

The present investigation gave repeated indications that changes in distensibility and/or contractility of the myocardium take place in the

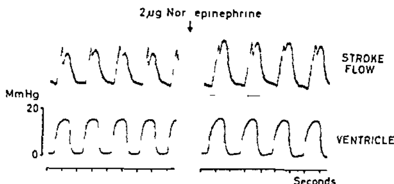


FIGURE FORTY ONE

The response in stroke flow and intraventricular pressure to injection of 2 µg nor epinephrine The intraventricular pressure increased moderately while the stroke volume rose from 0 26 ml to 0 41 ml There was a net increase in cardiac output from 8 85 ml/min to 12 3 ml/min Time marks one second

lower vertebrate heart as well Figure 41 demonstrates the effects on ventricular outflow and pressure from injection of 2 µg nor epinephrine into a mesenteric vein The stroke volume increased conspicuously from *0 26 ml to 0 41 ml* There was a slight reduction in heart rate while there was a net increase in cardiac output from 8 85 ml/min to 12 30 ml/min

DISCUSSION

No earlier results on cardiac output and simultaneous intracardiac and intravascular pressures in a lower vertebrate have to the author s knowledge been published A discussion and evaluation of the data will therefore be an attempt to put the acquired *information in proper* relation to modern views on cardiac performance and regulation in higher vertebrates in general, notably a selected group of mammalian species

most efficient functioning A small heart has to contract through a wider range than a large heart in order to expel the same volume However, the strain on a muscle fiber in holding a high pressure or in rapidly ejecting a given volume is concurrently smaller (Laplace law) The atria in *Amphiuma* seem in a unique way to have taken advantage of both these physical factors The volume of the atrium (right atrium) is generally large and a relatively small shortening will expel a sizable volume A considerable portion of the atrial volume however, is made up of the finger like muscular diverticula of very small dimensions These spaces may contribute to the rapid ejection and development of tension without necessitating a large strain on their muscle fibers [1]

E VENTRICULAR END DIASTOLIC VOLUME AND VENTRICULAR DISTENSIBILITY

As has been emphasized earlier the ventricular end diastolic volume in *Amphiuma* is the result solely of atrial activity combined with possible concomitant changes in ventricular distensibility Distensibility is here taken to imply changes in the resistance to stretch of the myocardium The atria in *Amphiuma* are hence given a double task namely to act as receiving and storing chambers and at the same time by their contraction to be responsible for the degree of ventricular distension and hence the energy release of ventricular contraction in accordance with the Starling concept The results demonstrated however, that the diastolic fiber length was not the sole determinant of the stroke volume Thus the stimulus of an increased backflow was not always sufficient to bring about an increased cardiac output Both the general state of the myocardium and the size of the residual volume seemed to exert an effect on the shortening of the ventricular myocardium Likewise it was frequently observed that an increased stroke volume could prevail for a considerable time, even at smaller heart sizes when the ventricle adjusted to a smaller systolic residue The resistance of the ventricle to the inflowing blood seemed similarly to be an independent variable This was disclosed by drug injections (acetylcholine epinephrine) when a larger end diastolic volume could ensue Changes in distensibility and amount of systolic residue must therefore be considered important modifying factors for the end diastolic fiber length and the stroke volume discharged also in *Amphiuma* Shannon and Wiggers (1939) maintain that the cardiac reserve represented by the large residual volume in frogs differs widely from conditions in mammalian forms where the residual volume is relatively far smaller They argue that both the turtle and frog retain

[1] This idea is the outcome of discussions with Dr R Hol of the Radiology Department at Ullevaal Hospital Oslo Norway

for representatives of all classes of lower vertebrates A considerable storage factor is represented in the sinus venosus of *Amphiuma* The reservoir is particularly accessible for instant mobilization The fact that both rate and force of the sinus venosus contractions may be markedly influenced by signals of an increased filling pressure from the greater veins seems of fundamental consequence

C ATRIAL END DIASTOLIC VOLUME AND ATRIAL DISTENSIBILITY

The aforementioned importance of the atria for cardiac performance in *Amphiuma* puts particular emphasis on the atrial end diastolic volume and its determinants It seems of importance that atrial filling to a large extent takes place as a result of an active contraction in the sinus venosus The atrial structure thus appears to be highly compliant This is a factor of possible importance for its filling, considering the changes in intrapericardial pressure set up during ventricular contraction These pressure changes will likely affect the atrial attraction or acceptance to the inflowing blood

The large spontaneous changes in end systolic or residual volumes observed in the atria, particularly in the right, provide further evidence of the important role played by these compartments in the total cardiac energy release

D ATRIAL CONTRACTILITY

It was clearly demonstrable from the radiological experiments (Part Three) that ventricular filling is an extraordinarily rapid process occupying considerably less time than it would in a mammalian heart at comparable heart rate The impedance to ventricular inflow must be extremely low as the atrial contraction only rarely appeared in the ventricular pressure record The almost explosive filling of the ventricle also points toward a very high rate of change in length of the atrial muscle fibers An increase in the rate of change in length and the rapid development of tension is exactly what is covered by the term 'increased contractility' in the present context This term does not have a generally accepted definition and much confusion has arisen from its diffuse meaning Rushmer (1961) maintains that increased contractility means that the myocardium develops tension more rapidly, shortening occurs more quickly and ejection is faster, without a necessary accompanying increase in the actual quantities of these variables The stroke volume can hence be increased by increased contractility, in particular by more complete systolic ejection

The peculiar atrial structure referred to earlier in this discussion invites some speculations about the optimal size of a cardiac chamber for its

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The peculiar atrial structure referred to earlier in this discussion invites some speculations about the optimal size of a cardiac chamber for its

pressure Their results evoke an obvious objection in that an artificially increased heart rate such as they used is radically different from a tachycardia governed by an increased load on the heart arising from normal conditions

An increase in the load on the heart produced by an artificial increase in venous return as applied in the present study is similarly not comparable with normally occurring exercise or similar conditions demanding an increased output from the heart However, an increase in the venous return is a necessary accompaniment to any adjustment to a maintained higher level of cardiac output

The responses obtained varied greatly, and even in one specimen two successive attempts to artificially increase the venous return could give opposite results one a tachycardia with a decreased stroke volume and one an increase in stroke volume with unchanged heart rate No conclusive evidence became available indicating the factors responsible for each response There are indications that a heart with a large end diastolic size responded with no change in heart rate

Meanwhile it was conclusively demonstrated that a significant increase in cardiac output was always associated with an unchanged heart rate and increased stroke volume The decreased stroke volume during tachycardia was never compensated for by the increased heart rate and cardiac output remained unchanged or increased insignificantly This finding is at variance with results obtained in mammalian forms where an increase in stroke volume is not an essential feature of the cardiac response to exercise (Franklin *et al* 1959)

The stronger emphasis on stroke volume for an increased output in the lower vertebrates is most marked in fishes, where the heart seems to lack sympathetic innervation entirely (Stannius 1849) and rarely responds with a tachycardia to an artificial increase in venous return (Johansen 1962)

SUMMARY

Pulsatile cardiac outflow and intracardiac and intravascular pressures have been recorded simultaneously in *Amphiuma tridactylum*

An increased venous return or outflow resistance to the sinus venosus and atria produced augmentation in both rate and force of contraction of these chambers Atrial contraction coincided with the period of ventricular filling and was entirely responsible for the latter Ventricular contraction lasted long compared to conditions in higher vertebrates The timing and ejection pattern from the heart is described

In a 750 g specimen the stroke volume was 0 67 ml, giving a cardiac

a substantial residual volume at the end of each ejection, sufficient to cause a *significant* ventricular discharge in a premature contraction beginning during the isometric relaxation phase. The importance of the systolic residue as a regulating factor has more recently become accepted teaching in mammalian cardiovascular physiology, thanks to a number of workers (Rushmer 1955, Katz 1955, and Gauer 1955). The difference between the lower and higher vertebrates in this respect is hence a matter of degree. In the present context it can be said that the residual volumes *of all cardiac compartments in Amphiuma* seem to participate in the instantaneous regulation of cardiac output.

F. VENTRICULAR CONTRACTILITY

The results obtained with injections of cathecolamines demonstrated an explicit change *in contractility with only minor changes in the actual* levels of pressure reached in the ventricle. The slope of the pressure gradient dp/dt was used as a measure of changes in contractility.

G. THE CONTRACTION OF THE BULBUS CORDIS

The *principal role of the contractile, highly compliant* bulbus segment is to act as a pressure chamber (Part Four). This in turn makes the bulbus *responsible for the maintenance of diastolic pressure* and a long lasting outflow period from the heart. The data obtained offer additional suggestive evidence that the bulbar contraction may add volume to the ventricular discharge.

H. ARTERIAL PRESSURE

The prevailing level of pressure in the systemic and pulmonary circuits *will modify the ventricular output by changing the resistance to outflow*. The relative changes in diastolic pressure in the two circuits set up by *vasomotor reactions will be a major factor in the adjustment of volume* flow in the two circuits.

It was demonstrated that an *increase in peripheral resistance brought* forth by lifting the tail represented a strong stimulus to an increased ventricular force of contraction.

I. HEART RATE

Shannon and Wiggers (1939) concluded from their studies on turtles and frogs that these animals, unlike mammals, do not increase the minute output by an increase in heart rate. They noted that the normal heart rate was optimal in the sense that a further increase in rate caused a progressive reduction in minute volume with decline in systolic blood

most efficient functioning A small heart has to contract through a wider range than a large heart in order to expel the same volume However, the strain on a muscle fiber in holding a high pressure or in rapidly ejecting a given volume is concurrently smaller (Laplace law) The atria in *Amphiuma* seem in a unique way to have taken advantage of both these physical factors The volume of the atrium (right atrium) is generally large and a relatively small shortening will expel a sizable volume A considerable portion of the atrial volume, however, is made up of the finger like muscular diverticula of very small dimensions These spaces may contribute to the rapid ejection and development of tension without necessitating a large strain on their muscle fibers [1]

E VENTRICULAR END DIASTOLIC VOLUME AND VENTRICULAR DISTENSIBILITY

As has been emphasized earlier the ventricular end diastolic volume in *Amphiuma* is the result solely of atrial activity combined with possible concomitant changes in ventricular distensibility Distensibility is here taken to imply changes in the resistance to stretch of the myocardium The atria in *Amphiuma* are hence given a double task namely to act as receiving and storing chambers and at the same time by their contraction to be responsible for the degree of ventricular distension and hence the energy release of ventricular contraction in accordance with the Starling concept The results demonstrated however, that the diastolic fiber length was not the sole determinant of the stroke volume Thus the stimulus of an increased backflow was not always sufficient to bring about an increased cardiac output Both the general state of the myocardium and the size of the residual volume seemed to exert an effect on the shortening of the ventricular myocardium Likewise it was frequently observed that an increased stroke volume could prevail for a considerable time even at smaller heart sizes when the ventricle adjusted to a smaller systolic residue The resistance of the ventricle to the inflowing blood seemed similarly to be an independent variable This was disclosed by drug injections (acetylcholine epinephrine) when a larger end diastolic volume could ensue Changes in distensibility and amount of systolic residue must therefore be considered important modifying factors for the end diastolic fiber length and the stroke volume discharged also in *Amphiuma* Shannon and Wiggers (1939) maintain that the cardiac reserve represented by the large residual volume in frogs differs widely from conditions in mammalian forms where the residual volume is relatively far smaller They argue that both the turtle and frog retain

[1] This idea is the outcome of discussions with Dr R Hol of the Radiology Department at Ullevaal Hospital Oslo Norway

for representatives of all classes of lower vertebrates A considerable storage factor is represented in the sinus venosus of *Amphiuma* The reservoir is particularly accessible for instant mobilization The fact that both rate and force of the sinus venosus contractions may be markedly influenced by signals of an increased filling pressure from the greater veins seems of fundamental consequence

C ATRIAL END DIASTOLIC VOLUME AND ATRIAL DISTENSIBILITY

The aforementioned importance of the atria for cardiac performance in *Amphiuma* puts particular emphasis on the atrial end diastolic volume and its determinants It seems of importance that atrial filling to a large extent takes place as a result of an active contraction in the sinus venosus The atrial structure thus appears to be highly compliant This is a factor of possible importance for its filling, considering the changes in intrapericardial pressure set up during ventricular contraction These pressure changes will likely affect the atrial attraction or acceptance to the inflowing blood

The large spontaneous changes in end systolic or residual volumes observed in the atria, particularly in the right, provide further evidence of the important role played by these compartments in the total cardiac energy release

D ATRIAL CONTRACTILITY

It was clearly demonstrable from the radiological experiments (Part Three) that ventricular filling is an extraordinarily rapid process occupying considerably less time than it would in a mammalian heart at comparable heart rate The impedance to ventricular inflow must be extremely low as the atrial contraction only rarely appeared in the ventricular pressure record The almost explosive filling of the ventricle also points toward a very high rate of change in length of the atrial muscle fibers An increase in the rate of change in length and the rapid development of tension is exactly what is covered by the term 'increased contractility' in the present context This term does not have a generally accepted definition, and much confusion has arisen from its diffuse meaning Rushmer (1961) maintains that increased contractility means that the myocardium develops tension more rapidly, shortening occurs more quickly, and ejection is faster, without a necessary accompanying increase in the actual quantities of these variables The stroke volume can hence be increased by increased contractility, in particular by more complete systolic ejection

The peculiar atrial structure referred to earlier in this discussion invites some speculations about the optimal size of a cardiac chamber for its

PART SIX

An experimental evaluation of the double circulation in *Amphiuma tridactylum*

The transformation from a single circulation in fisches to the complete double circulation with separated systemic and pulmonary circuits in post embryonic birds and mammals is a major event in the phylogeny of vertebrates In fact very few phenomena have had such decisive influence on modern views of vertebrate evolution as the development of the heart and great vessels

Until recently the subject has been treated exclusively from an anatomical viewpoint and although physiological implications have been discussed the inference has most often been solely from anatomical deductions Experimental data recorded with adequate physiological technique are very scarce This holds particularly true with reference to conditions within the clas s *Amphibia* The question whether or not a selective distribution of blood on its passage through the heart exists in amphibians is hence subjected to sharp differences of opinion

The existing controversy is complicated by the uncertain position of modern *Amphibia* in a direct evolutionary line from the piscine condition to the higher tetrapods Problems of specialization, regression and convergence add difficulties to the discussion From the point of view of circulatory adaptations to different modes of respiration the *Amphibia* provide an exclusive class of the utmost importance

The present investigation is an attempt to produce experimental evidence about the pattern of circulation through the heart of an amphibian By selecting a large species it has been possible to apply a hitherto unattempted methodical approach to the problem This involves analysis of the blood oxygen content in the incoming vessels to the heart compared with the corresponding conduits leaving the heart Apart from a preliminary report (Johansen 1962), such analysis has never been recorded for any species of *Amphibia* in spite of the fact that such procedures have put an end to the discussion about mixing conditions in the reptilian heart (White 1956 1959 Steggerda and Essex 1957)

The present investigation also contains data obtained with high speed cinefluorography and film changer technique as well as simultaneous measurement of intravascular pressures in the systemic aorta and pulmonary circuit

output of about 30 ml/kg/min An artificial increase in venous could evoke an increase in the stroke volume with no change in rate, or a marked tachycardia with reduced stroke volume In the case the cardiac output remained largely unchanged, whereas creased stroke volume gave a significant increase in cardiac outpu residual volumes in the atria and ventricle represented an imp cardiac reserve Factors other than the end diastolic volume, su changes in distensibility and contractility, were important in the lation of the stroke volume

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ration with pure oxygen If cutaneous respiration was restricted by drying the skin the measured arterio venous differences in oxygen content increased significantly Blood returning from the skin is drained in the sinus venosus and contributes only to a general elevation of the blood oxygen content

Radiological examinations

A primary requisite for a selective distribution of blood on its passage through the amphibian heart is to keep the two types of inflowing blood separate in the undivided ventricle In order to elucidate this problem contrast was injected in the posterior vena cava and followed by serial roentgen exposures on its passage through the heart

Figure 43 demonstrates an early stage of right atrial contraction subsequent to contrast injection in the posterior vena cava Atrial con traction had closed the sino-atrial valves opening the atrio ventricular valves and the ventricle is seen to be filled in its right segment The filling occurred in discrete furrows arranged in antero posterior direction

Figure 44 is a series of roentgen frames demonstrating the passage of contrast injected into the posterior vena cava The animal was placed in dorsal position The contrast injection filled the right atrium (Fig 44A) One frame later (0 50 sec) the rapid atrial contraction filled the right portion of the ventricle leaving a distinct demarcation toward the left side (Fig 44B) During the subsequent ventricular contraction (Fig 44 C D E) the clear demarcation between the right and left portion of the ventricle was maintained both in the phase of isometric contraction as well as throughout the entire period of ventricular ejection Details about the course of the ventricular discharge up through the bulbus cordis and on to the various arterial arches were somewhat obscured by the next atrial filling covering the same area on the films It is, however beyond doubt that there existed a clear preference for filling of the pulmonary arteries Frame D in Figure 44 shows that the contrast had propagated past the arch in the truncus arteriosus and in a posterior direction to a level corre ponding to the apex of the heart At the same time there was only a vague filling of one of the aortic trunks of the truncus arteriosus In the subsequent frame (Fig 44E) the pulmonary arteries are well defined along their entire course while only the right aortic arch is faintly defined

Figures 45 and 46 depict corresponding series of roentgen films with the contrast medium injected in the pulmonary vein Figure 45A shows a simultaneous filling of the veno atrial and the atrio-ventricular junction The ventricle is filled in discrete furrows in the upper left section It should be noted again that the increase in intraventricular pressure

RESULTS

Table 1

Oxygen content of blood from the great vessels of *Amphiuma tridactylum*

Animal No	O_2 cont (vol %) Pulmonary arch	O_2 cont (vol %) Aortic arch	O cont (vol %) Sinus venosus	O cont (vol %) Pulmonary vein	Method
1	4 8	7 7			Natelson (micro van Slyke)
2	5 3	11 5			
3	6 5	10 1			
4	4 8	6 7			
4	5 3	8 2		8 4	
5	6 3	8 6			Scholander
5	6 8	8 1			
6	6 1	6 3			
7	6 4	10 5			
7	6 9	8 5	5 0	8 5	
8	8 8	8 8			
9	7 9	8 6			,
9	6 8	8 1	6 7	7 6	
10	9 6	11 0			
10	9 0	10 0	6 8	10 5	,
11	7 5	9 1			
12	8 2	10 2	7 9	10 2	,
13	8 5	10 2	5 0	10 2	

Oxygen Analysis

Table 1 summarizes the results obtained from the analysis of oxygen content in the great vessels of *Amphiuma tridactylum* In animals 7, 12, and 13 there is a complete correspondence in oxygen content between blood from the pulmonary vein and the aortic arch In animal 12 the correspondence extends to blood from the sinus venosus and pulmonary arch respectively In animal 13, however, the blood oxygen values in the pulmonary artery are increased compared to the values from the sinus venosus

In other animals studied there was always some degree of mixing The selective passage could easily be disturbed or abolished, as can be seen from animals 6 and 8 A factor of prime importance was the general level of pressure in the heart, bulbus cordis, and various arterial arches At low pressures (and hemorrhage, animals 6 and 8) an almost complete mixing seemed to ensue

It is interesting to note the small arterio venous differences in oxygen content The difference remained small, even following lung respi-

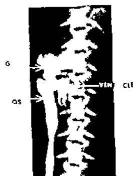


FIGURE FORTY THREE

A contrast injection in the posterior vena cava demonstrates the inflow pattern from the right atrium into the right portion of the ventricle. Animal in ventral position. Exposure at 50 Kv. Exposure time 0.03 seconds.

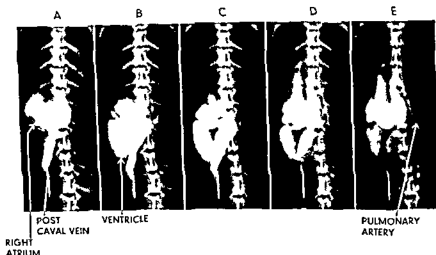


FIGURE FORTY FOUR

Contrast injection in the posterior vena cava. Note the clear demarcation between the right and left portion of the ventricle. The selective distribution is largely maintained in the bulbus cordis with a preference for filling of the pulmonary arteries. Animal in dorsal position. Rate of exposures 2 frames per second at 50 KV. Exposure time 0.03 seconds. A, B, C, D, E are consecutive frames.

during ventricular contraction did not deteriorate the selective ventricular filling (Figs 45 and 46) At maximal ventricular contraction (Fig 45E) the residual ventricular content was still arranged in furrows with no admixture to the right side Figure 46, taken with the animal in a slightly oblique ventral position, reveals the pattern of ventricular outflow through the bulbus cordis The contrast is seen to be expelled in well defined streams through the partially divided bulbus cordis Reaching the truncus arteriosus, which is completely divided into systemic and pulmonary conduits, the sharp laminar outflow pattern is dissolved and defines the large caliber of the systemic conduits of the truncus arteriosus Figures 45 and 46 both give a manifest demonstration of a high degree of selective filling from the left atrium through the undivided ventricle and bulbus cordis and on to the systemic arterial arches

To describe more closely the pattern of ventricular outflow a series of contrast injections were done with roentgen exposures taken with a horizontal beam By doing this it was possible to avoid a large extent of overlapping between the atrium and the bulbus cordis In the experiment underlying Figure 47 the contrast medium was injected into the pulmonary vein The left atrium and the ventricle as well as the bulbus cordis and truncus arteriosus leading off into the various arches are well defined In the proximal part of the bulbus cordis the ventricular discharge is arranged in two separate streams Where the bulbus makes a bend toward the left, the two streams depart, each making a loop The most ventral stream makes a sharp loop and passes dorsally in the anterior direction after having crossed the other stream In the truncus arteriosus this stream comes to lay dorsally, but later makes an acute bend in the posterior direction The bend represents one pulmonary arch leading off into one of the two pulmonary arteries The other stream makes a loop and passes ventrally in the anterior direction (Fig 47E) and on to the truncus arteriosus, where it splits up into paired aortic and carotid trunks In the occipital region they turn dorsally, and the two aortic arches unite to form the dorsal aorta in the posterior direction

In the truncus arteriosus there is complete anatomical separation between the various systemic and pulmonary arterial trunks In the bulbus, however, the anatomical structures, including the spiral valve, offer guidance to the two streams, although there is ample opportunity for admixture Figure 48 demonstrates how the discrete, streamlined outflow pattern extends far into the contracting ventricle When too large volumes of contrast were injected the selective distribution was reduced or abolished

A

B

C

D

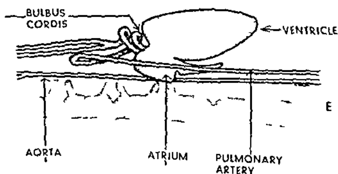


FIGURE FORTY SEVEN

Contrast injection in the pulmonary vein *Animal in* dorsal position Roentgen exposures in side projection (horizontal beam) *The outflow* pattern from the ventricle to the various arterial arches is demonstrated *The* schematic drawing illustrates the helical motion of the outflow streams when *passing through* the bulbus cordis Rate of exposures *2* frames per second at 50 KV Exposure rate 0 03 seconds *A* and *B* are consecutive frames *BC* and *CD* are spaced one second apart

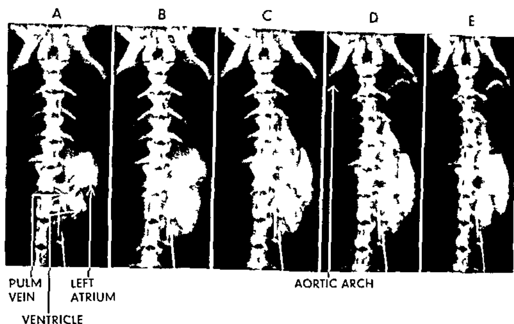


FIGURE FORTY FIVE

Contrast injection in the pulmonary vein Frame A shows the left atrium with veno-atrial and atrio ventricular junctions Ventricular filling is restricted to the upper left portion of the ventricle (frames A and B) Frames C D E demonstrate a clear preference for filling of the systemic arteries Animal in dorsal position Rate of exposures 2 frames per second at 50 KV Exposure time 0 03 seconds A B C D E are consecutive frames

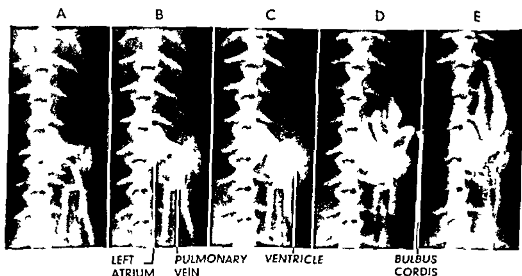


FIGURE FORTY SIX

Contrast injection in the pulmonary vein demonstrates the *streamlined* outflow pattern from the contracting ventricle through the *undivided* bulbus cordis and on to

Pressure measurements

The simultaneous recording of pressures in one pulmonary artery and a systemic artery gave a rather wide range of pressures in different animals The systolic pressure values were always quite similar in the two circuits, while the diastolic pressures varied greatly, particularly in the pulmonary circuit, which could display diastolic pressures similar to those recorded

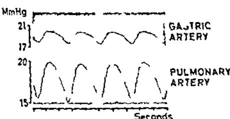


FIGURE FORTY NINE
Simultaneous pressure recordings from a branch of the gastric artery and a pulmonary artery
Time marks onesecond

in the systemic circuit or markedly lower Figure 49 demonstrates a situation where the pressures in the gastric artery and a pulmonary artery were recorded simultaneously, cannulated at equal distance from the heart The results were checked by interchanging the pressure transducers The systolic values are quite similar, and fluctuate around 20 mm Hg The diastolic levels however were decidedly lower in the pulmonary artery Note also the more abrupt and rapid rise and fall of pressure in the pulmonary circuit There is a slightly earlier pressure rise in the pulmonary artery Their maxima however, are reached at the same time

DISCUSSION

The long lasting discussion about the mode of circulation through the amphibian heart is centered around an old account of the circulation through the heart of the frog (Brucke 1852, later modified by Sabatier 1873) now referred to as the classical hypothesis In brief this theory states that oxygen rich blood returning from the lungs through the pulmonary veins to the left atrium will occupy the left portion of the ventricle whereas the oxygen depleted blood returning through the sinus venosus to the right atrium will come to occupy the right portion of the ventricle It was alleged that the trabeculate structure of the ventricle tended to keep the two types of blood unmixed in the ventricle Upon ventricular contraction the oxygen poor blood occupying the right portion of the ventricle was assumed to leave the ventricle first because of a closer situation of the bulbus aperture to the right of the ventricle The bulbus cordis is partially divided by a spiral valve Aided by this valve the oxygen poor blood was directed toward the pulmonary

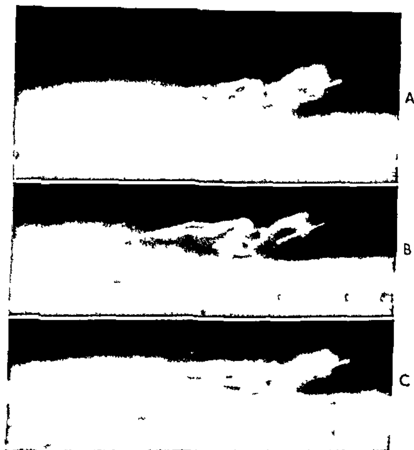


FIGURE FORTY EIGHT

Contrast injection in the pulmonary vein Animal in dorsal position Roentgen exposures in side projection (horizontal beam) The typical channeling of the ventricular discharge extends far into the contracting ventricle In frame C the aorta is well defined while there is no filling of the pulmonary arteries past the pulmonary arches Rate of exposure 2 frames per second at 50 KV Exposure rate 0 03 seconds A and B are consecutive frames C falls 1 5 seconds later

flow conditions within the heart became clear as relatively slight hemorrhage and weakening of the heart abolished the selective distribution

The experimental series is too limited to document a definite trend in the shunting pattern The six experiments with values obtained on the venous as well as on the arterial side of the heart suggest that a left to right shunt is prevalent The samples from the sinus venosus were taken posterior to the entrance of the left and right ducts of Cuvier As these vessels carry blood from the pharyngeal region, the oxygen values in the right atrium were possibly slightly higher

Steggerda and Essex (1957) demonstrated the pattern of circulation in the turtle heart (*Chelhydra serpentina*) by oxygen analysis They emphasize the dominance of a left to right shunt (50 80%) compared to a small (5 10%) right to left shunt They point out the similarity between this finding and what is commonly encountered in mammals with large congenital ventricular septal defects

The small arteriovenous oxygen differences found in the present study serve to emphasize the importance of cutaneous and pharyngeal respiration in *Amphiuma*

Most studies dealing with the mode of circulation through the amphibian heart utilize some form of indicator technique based on the injection of a substance that can be traced along its course through the heart to the larger arteries

Most of these techniques suffer serious deficiencies and great caution must be observed in the interpretation of the results If, however the pressure and volume relations on the venous side are not unduly disturbed by the injection of an indicator and provided the surgical interference necessary to study the indicator s passage through the heart is not too disturbing the method should disclose whether mixing takes place within the undivided ventricle and bulbus The method will, however, be of less use in the evaluation of the extent of admixture between the left and right side

Ozorio de Almeida (1923) studying the large South American bullfrog *Leptodactylus ocellatus* found distinct demarcations between the two types of blood in the ventricle when he applied artificial lung respiration As soon as the respiration was suspended the demarcation disappeared promptly He observed the heart *with direct illumination* As early as 1869 Fritsch had argued that the color changes that can be observed in this way are due entirely to differences in the transparency of the vessel walls Similarly Foxon (1947) maintains that observations from a visual inspection in direct light have no obvious relationship to the blood inside but merely reflect varying shades of red ascribable to the vessel

artery This directional flow was also thought to be assisted by a lower pressure existing in the pulmonary vessels As ventricular contraction proceeds and the pressure increases the spiral valve was assumed to change position, shutting off the flow to the pulmonary artery, with a consequent channeling of blood into the aortic arches This blood was called mixed blood, and was assumed to have occupied the middle portion of the ventricle Finally, toward the end of ventricular contraction the purely oxygen rich blood would enter the carotid arches ensuring the supply of only oxygen rich blood to the brain The reasoning for this last separation was based on the idea that pressure in the carotid labyrinths had to be overcome before blood could be expelled into the carotid arteries This 'classical hypothesis' was accepted early and has been the general teaching, even in modern textbooks

Vandervael (1933) discarded the hypothesis as completely erroneous and produced evidence of a randomized distribution of blood in the amphibian heart His work made the basis of the so called 'mixing hypothesis' More recent accounts have not conclusively solved the problem

The major part of the studies published to elucidate the pattern of the circulation in amphibians deals with anurans, and species of the genus *Rana* have aroused particular interest Few earlier experimental studies on the urodele amphibians have been published (Noble 1925) Foxon (1955) has pointed out that when considering recent *Amphibia* it should be noted that the *Anura* and and *Urodela* differ from each other in many respects, and that the frogs and the salamanders are descended from 'very different Palaeozoic stocks' (Romer 1945)

In the present context differences between anurans and urodeles are of secondary importance, as the main objective is to describe the status and possible mechanisms of a double circulation without considering its phylogenetical relationship On this basis a comparison with anurans is justifiable, particularly because the anatomy of the central cardiovascular structures in *Amphiuma* shows many similarities with conditions in frogs The mode of respiration with primarily lung breathing but also pharyngeal and cutaneous breathing shows small variance from conditions in *Rana* (Baker 1949)

No available method can bring more conclusive evidence to the question of mixing conditions than analysis of the blood oxygen content in the greater vessels connected with the heart The data presently obtained bring indisputable support to the idea that the amphibian heart, represented by *Amphiuma tridactylum*, can accommodate two separate blood streams with only slight mixing occurring under certain circumstances That such a selective distribution depends closely on normal pressure and

which de Graaf maintains is the larger of the two will to a considerable extent contaminate the right ventricular blood De Graaf is unable to offer any explanation as to how the selection is maintained in the bulbus He feels that the larger circulating volume in the pulmonary circuit is a natural consequence of the lesser peripheral resistance in that direction The smaller resistance in the pulmonary circuit he evaluated from the low diastolic pressure levels in these vessels From the form of the pulse curve he deduced that the spiral valve blocks off the entrance to the pulmonary arch before the major propulsive force of the ventricular contraction is expended The significance of the spiral valve continues to remain obscure He suggests that its movement may ensure that the systemic vessels receive sufficient blood in spite of their greater peripheral resistance In a more recent study Simons (1959) arrived at conclusions equally distinct from the 'classical hypothesis although he defends a selective distribution He worked with the anurans *Rana temporaria* and *Bufo bufo* and the urodeles *Salamandra maculosa* and *Triturus cristatus* His methodical approach consisted in using the dye Evans Blue as an indicator and cinephotographic technique in recording the passage of the dye through the heart He drew the conclusion that the movement of the blood is simultaneous in all the arterial arches The contents expelled from the two atria remained virtually separate in the passage through the ventricle as far as the base of the bulbus cordis (conus arteriosus) From there on the separation was maintained in the anurans by the presence of the spiral valves whereas mixing took place in the two species of urodeles because of the reduction or lack of a spiral valve

The larger size of *Amphiuma* compared to amphibians studied earlier gives a definite advantage in the application of an indicator technique The volume of contrast injected was small compared to the total inflowing volume The injection pressure was similarly kept low It should be noted however that the contrast medium used Hypaque 45% has a fairly strong vasodilatory effect in mammals (Lindgren and Tornell 1958) The procedure involved a minimum of surgery with no direct interference to the heart itself Cineradiography and film changer techniques gave a direct recording of the phenomena independent of the investigator s visual observations Figures 44 45 and 46 leave no doubt that atrial inflow is largely kept separated in the undivided ventricle The trabeculate sponge like structure of the ventricle seems to be mainly responsible for this by receiving the blood from the two sources into the numerous slit like chambers in the ventricular wall The effectiveness of such means for separation is stressed when it is remembered that the selective distribution may also be retained during the entire ventricular systole The author contends that in order to achieve this high degree of

walls Ozorio de Almeida's observations clearly refute the statement of Foxon, in particular because the color changes were also apparent in the ventricle and were clearly related to the artificial respiration Foxon's approach to the problem was to use the transillumination method devised by Vandervael (1933) Vandervael, in his work on *Rana (temporaria and esculenta)*, had not only discarded the explanation for selective distribution in accordance with the 'classical theory, but he denies that a selective distribution takes place at all He found no time difference in the movement of blood in the carotid, aortic, and pulmo cutaneous arteries, and that blood coming into the ventricle from the two atria was mixed completely in the ventricle and not kept separated Noble (1925), working with a large number of species, both anurans and urodeles, had used ink solutions and followed their pathways visually through the heart and great vessels He gave his full support to the 'classical theory' for the species that use their lungs as the *most important respiratory mechanism* and have a correspondingly well developed spiral valve arrangement in the bulbus cordis Peelle (1931) and Acolat (1931, 1938 a, b) similarly used dye injections and obtained separation of the streams, although Acolat *admits that some mixing must take place Foxon and Walls (1947), in* agreement with Vandervael, maintain that the distribution in the ventricle is *considerable*

Hazelhoff (1952), working with *Rana*, and Simons and Michaelis (1953), working *with the Australian frog, Hyla caerulea, found* a selective distribution The latter authors used ultraviolet illumination and injections *of a fluorescent dye into the venous system* They *found the* selective distribution a very labile characteristic depending on circumstances which *had yet to be defined Apart from the meticulous* works of Foxon (1947, 1951), Simons and Michaelis (1953), de Graaf (1957), and Simons (1959), *most workers have approached the problem* largely in a speculative way, *with few experimental verifications of their suppositions* This is *true also of* Sharma (1957), who worked with *Rana tigrina* and deduced a selective distribution from purely anatomical studies He is, however, *the only author to report the existence of a* particular ventricular septum in an amphibian He is not referring to an extension of the atrial septum *into the* ventricular lumen, but to a muscular ridge existing as a separate entity De Graaf (1957) presented an experimental study on *Xenopus laevis*, dealing with the blood distribution Like Simons and Michaelis, he used injection of fluorescein as indicator He concludes that there must be a physiological connection between the right side of the ventricle and the pulmo cutaneous arches and between the left side of the ventricle and the other arches However, although the right atrial blood does not contaminate the left side of the ventricle, the output of the left atrium,

laminar flow through the bulbus cordis The retention of the discrete, separate paths through the entire bulbus and into the arterial conduits of the truncus arteriosus strongly suggests that some form of anatomical guidance supports the laminar outflow pattern, especially as the two paths make a full loop without losing their separate course The spiral valve as well as the bulbar in and outflow valves assumingly participate in this anatomical support The films did not disclose whether the filling of the pulmonary arches preceded or was simultaneous with the filling of all the arterial arches

Figure 48 shows that following a contrast injection in the pulmonary vein the systemic arches are visible throughout the bulbus and truncus, and continue as the dorsal aorta far back in posterior direction whereas the pulmonary circuit is only faintly discernible past the arch This may indicate that the separating mechanism at this level may involve an obstruction in the bulbus possibly related to the position of the spiral valve When a heart was working at a large size with a relatively large stroke volume the degree of mixing seemed to increase

The lower resistance in the pulmonary circuits and the role of this in the separating mechanism is frequently stressed in the literature Only a few workers however have measured the pressure difference in the two circuits and nobody has earlier conducted measurements on intact free moving specimens

Gomperts (1884) working with frogs, found no difference in pressure, but that there was a simultaneous rise and fall of pressure in the three arches Acolat (1938a b) found a slightly lower pressure (1 3 mm Hg) in the pulmonary artery of frogs He noted that the pressure rose and fell more abruptly in the pulmonary artery De Graaf (1957) recorded simultaneous pressures with adequate techniques He found wide ranges in pressure in some experiments ascribable to the level of anesthesia The main feature of his results was that the pulmocutaneous artery showed consistently lower pressure than those in the carotid or aortic arches The pulmonary artery pressure was most commonly 7 mm Hg lower in diastolic levels compared to the others The rise and fall of pressures in the pulmonary artery were invariably more rapid than the others and he found a slight but true delay in the beginning of the pressure rise in the systemic arch (ca 0 1 sec) Simons (1957), on the other hand found on the average no difference in pressure between the pulmonary and systemic circuits although the individual pressure levels were very labile

The pressures recorded in the present study present an extremely variegated picture particularly in the awake free moving animals The author fully believes that these variations were not circumstantial but reflect normal events in the pressure levels It is generally appreciated

selective distribution, the marked antero posterior trabeculation of the ventricular lumen is of paramount importance The trabeculation will not only tend to keep the two types of blood separate during diastole, but as systole begins the shortening of the muscle fibers will close the free ventricular lumen and prevent admixture during isometric contraction When the bulbar valves open, the ventricular content will escape with a high initial velocity in two main streams formed by the numerous enclosed crypts The antero posterior orientation of the trabeculation inside the ventricle is probably a main factor in setting up the discrete laminar outflow, which makes a right-hand spiral movement at the exit from the ventricle and is guided higher up in the bulbus by the anatomical support of the bulbar valves The tendency of the slit like chambers to be arranged in the longitudinal axis of the heart gets stronger closer to the ventricular exit, where the muscular trabeculae form a main directional outflow avenue (Zullich 1930) The central cavity of the ventricle is small, even smaller than in the frog, and does not extend any distance caudal to the atrioventricular opening (Benninghof 1921) The present radiological data offer for the first time a recorded picture of the flow pattern through the entire amphibian heart, and justify the assumption that the ventricular structure described above forms the discrete streams seen during ventricular ejection The fact that under laminar flow conditions streamlines can exist as separate paths for long distances is well known from hydrodynamics Similar situations in hemodynamics have only sporadically been referred to It should be emphasized in this regard that Simons (1959) assumed that the contents of the ventricle as they are expelled into the bulbus are given a right handed spiral movement He feels that such a helical deflection will exert a directive effect and in a major way be responsible for the separation of the blood streams

It is of great interest to note that spiral streaming of a similar pattern has been reported to take place in the bulbar region of embryonic hearts Bremer (1932) described a double spiral streaming in the heart of the chick embryo Romhanyi (1952) and Goerttler (1955) observed a similar pattern in models of the embryonic human bulbus cordis Simons (1959) stated that the alleged helical motion of the blood passing from the ventricle in fully developed amphibians must be a property retained from the embryonic stages, where a helical arrangement of the muscle bundles prevails (Benninghoff 1933)

In Part Four of the present investigation a smooth long lasting outflow pattern from the heart of *Amphiuma* was described made possible by the pressure chamber action of the bulbus cordis It seems conceivable that the extended, smooth outflow may be a contributing factor to the maintained

Blood oxygen analysis radiological techniques, and intravascular pressure measurements have been used to evaluate the mode of circulation through the heart of *Amphiuma tridactylum* The blood oxygen values from the greater vessels arriving at and leaving the heart, as well as the radiological data, demonstrated a selective passage of blood from the right and left atria to the pulmonary arteries and systemic arteries respectively The selective distribution was very labile and could easily be abolished The trabeculate structure of the ventricle was an important separating factor The ventricular discharge showed a laminar outflow pattern with a right hand spiral movement through the undivided bulbus cordis The levels of blood pressure in the heart and larger vessels as well as the diastolic size and amount of systolic residue of the cardiac chambers were modifying factors in the selective passage Vasomotor activity in the pulmonary and systemic circuits will similarly influence the distribution of the ventricular discharge

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that the pulmonary vasculature is highly nonreactive both to neural and humoral stimuli, and is unaffected by the general vascular responses As a reason for this assumption (Rushmer 1961), it is argued that the lungs are the only recipient of the output from the right heart, hence there is no need for the distributing effect of vasomotor mechanisms This reasoning gives little thought to the phylogenetical development of the cardiovascular system In animals devoid of a complete double circulation, vasomotor activity must have decisive importance for pulmonary flow and hence the whole mixing problem Furthermore, it should be noted that the major cutaneous arteries (anurans) and arteries to the pharyngeal and esophageal region (urodeles) in amphibians are derived from the pulmonary arteries, which for this reason are often designated as pulmo cutaneous arteries The skin and pharyngeal region represent most important respiratory surfaces in amphibians, and distributing effects of vasomotor activity must be important Poczopko (1960) has shown from capillary counts *in vivo* that a deprivation of aerial breathing in frogs opens up a large number of cutaneous capillaries The same response could be evoked during graded hypoxia and aerial breathing He assumes a nervous control of the underlying vasomotor mechanisms Vasomotor activity in the skin or pharyngeal region will influence the overall pressure level in the pulmonary arch, which in turn will influence the distribution of volume flow in the systemic and pulmonary circuit Similarly, vasomotor changes in the systemic circuit will exert an effect on the pulmonary circuit

The diversity of the results obtained by the many workers on the mixing conditions in the amphibian heart can be attributed to the extraordinary lability of the factors responsible for a selective distribution These factors include the levels of pressure inside the heart and greater vessels as well as the size of the stroke volume and vasomotor reactions The mode of respiration may presumably also alter the circulatory pattern Most of these factors cannot be checked during experimental situations

The small arterio venous oxygen differences related to the multiple ways of respiration, and the lability of the selective distribution in itself suggest that the selection pressure for the establishment of a selective distribution of blood is only influenced to a limited extent by the oxygen needs of the tissues It seems possible that the mere presence of functional lungs provides the main stimulus in alterations of hemodynamical conditions in the direction of a functional double circuit in *Amphibia*



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PREFACE

The present work was carried out in the Department of Medical Chemistry University of Helsinki I am very much indebted to my honoured teacher and chief the late Professor P E SIMOLA M D Ph D for suggesting to me the subject of this investigation for his unfailing interest and advice and for his kindness in placing all the facilities of his department at my disposal

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Helsinki May 1963

AARNE RAINA

KESKUSKIRJAPAINO
HELSINKI 1963

CONTENTS

INTRODUCTION

Spermidine $H_2N(CH_2)_4NH(CH_2)_3NH_2$ and spermine $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH$ are aliphatic polyamines that have long been known to be normal tissue constituents However our knowledge of these amines their metabolism and biological function advanced relatively slowly until recent years

Studies concerning the occurrence of polyamines have shown that they are widely distributed in animal tissues Large quantities of spermidine and spermine are found for instance in the pancreas liver spleen and kidney Large amounts of spermine are found in human semen During the last few years polyamines have also been detected in several micro organisms and bacterial viruses The intra cellular localization of polyamines is poorly understood at present Evidence has been presented that certain intracellular particles *e g* ribosomes contain polyamines

Many reports have confirmed that polyamines are able to stabilize nucleic acids and nucleic acid containing particles such as ribosomes and viruses against degradation and certain denaturing agents This property taken with the occurrence of polyamines in the sub cellular particles has suggested that these substances may play a physiological role as stabilizers Further evidence is needed however to establish the correctness of this hypothesis

During the last decade much additional knowledge has accumulated concerning the metabolic fate of spermidine and spermine in micro organisms The degradation pathway has been elucidated in several bacteria *in vivo* and recently also using partially purified enzymes It has further been shown that certain isotopically labelled compounds are incorporated into polyamines The various steps in the biosynthesis of spermidine have also been confirmed with purified bacterial enzymes whereas the enzymic synthesis of spermine has not been successful at present

The metabolism of spermidine and spermine in animal tissues is as yet much less well understood There is only meagre evidence

REVIEW OF THE LITERATURE

Most of the early reports regarding the chemical, biochemical and pharmacological properties of spermidine and spermine have been reviewed in a monograph on the biogenic amines (GUGGENHEIM 1951). In the last ten years much additional knowledge respecting these amines has been accumulated. A review of the literature on the biochemistry of spermidine and spermine has recently been published by TABOR, TABOR and ROSENTHAL (1961). In the following section the various methods used for quantitative estimation as well as the occurrence, biosynthesis and degradation of these polyamines are described in broad outline. Some recent papers have also been cited which seem to afford a clue to the physiological role possibly played by spermidine and spermine in biological systems.

DETERMINATION OF SPERMIDINE AND SPERMINE

The older methods used for the quantitative determination of spermidine and spermine were gravimetric, based on isolation and purification of the polyamines as their poorly soluble salts, e.g. as phosphates, picrates or flavianates (for references and methods see GUGGENHEIM 1951). These precipitation procedures were time consuming and in most cases only semiquantitative, being generally more suitable for the estimation of spermine, because the corresponding spermidine salts, e.g. phosphate, are more soluble and therefore give poor yields. Further, for accurate determination large quantities of material were required.

For a reliable quantitative determination of spermidine and spermine a preliminary procedure is required to separate the polyamines from each other and from other contaminating substances, because the methods, and particularly the various colour reactions used for quantification are more or less unspecific. A review of the methods employed in the isolation and quantitative determination of polyamines is given below.

PURIFICATION AND SEPARATION OF AMINES FOR ASSAY

For isolation of spermine, butanol extraction and steam distillation from alkaline solution were used by DUDLEY, ROSENHEIM and ROSENHEIM (1924). In the purification of histamine butanol extraction had given good yields when an alkaline salt mixture was added to the aqueous phase (MCINTIRE, ROTH and

of the degradation of polyamines *in vitro* *In vivo* experiments however, have demonstrated that some breakdown does occur, although the degradation products are largely unidentified Similarly, practically nothing is known concerning the biosynthesis of spermidine and spermine in animal tissues

Studies on polyamines have been greatly hindered by lack of adequate analytical methods Thus a few years ago when the present work was started, the development of a reliable and sensitive method for quantitative determination was the first essential

The object of the present study was thus to develop a quantitative method for the determination of spermidine and spermine, which would allow estimation of small amounts of these amines The next problem was to study the metabolism of these amines in the developing chick embryo with special reference to their biosynthesis

In quantitative determinations of spermidine and spermine two reagents 2 4 dinitro 1 fluorobenzene and ninhydrin have mainly been used In addition *p nitroaniline* has al o been suggested for quantitative estimation (LAHDE VIRTA RAINA and HEIKEL 1957)

Since SANGER (1945) introduced 2 4 dinitro 1 fluorobenzene as a quantitative reagent for labelling the free amino groups in proteins and peptides this compound has found many applications It was shown for instance that dinitro fluorobenzene could be widely employed as a quantitative reagent for primary and secondary amines (McINTIRE CLEMENTS and SPROULL 1953) This permitted the d termination of quantities of the order of 0 1 μmoles of amine For estimation of spermidine and spermine the dinitrofluorobenz ne method was then use l by ROSENTHAL and TABOR (1956) Their method consisted of allowing the amine in sodium tetraborate buffer to react with dinitrofluorobenzene After acidification the dinitrophenyl d rivatives of amines were extracted with ethyl acetate methyl isobutyl ketone or cyclohexanone and determined photo metrically A further modification was developed by DUBIN (1960) who employed dioxane to bring the dinitrophenylamines into solution which made extraction with an organic solvent unnecessary He also reduced the volume of the reaction mixture According to DUBIN with these modifications his method was about ten times as sensitive as that of McINTIRE CLEMENTS and SPROULL (19 3) and four times as sensitive as that of ROSENTHAL and TABOR (1956) permitting accurate determination of 10 to 150 mμequivalents of amino group A simple te t based on differences in spectral properties of dinitro phenyl derivatives was also given for differentiating between primary and secon lary amines

It was reported by KOLBEZEN ECKERT and BRETSCHNEIDER (1962) how ever that with the several modifications of the 4 dinitrofluorobenzene method cited above they could not attain the accuracy and preci ion required for the quantitat e determination of some simple primary aliphatic amines To o erco n th difficulties er countered when any of the abo e mentioned methods was used uch as the formation of 2 4 dinitrophenetole or a high background they suggested several modifications to the procedure However exact information about the accuracy and sensitivity of the final method was not given

Ninhydrin is the other reagent commonly used in the quantitative deter mination of spermidine and spermine FISCHER and BOHN (1957b) de cribed a method for the determination of various amin s from tissue extracts after paper electrophoretic separation After electrophoresis the paper strips were dried in an air flow containing a minimum of ammonia then impregnated with ninhydrin solution and dried again The coloured amine fractions were then cut off and sprayed with copper nitrate solution as described by KAWERAU and WIELAND (1951) The red copper pigment was eluted with methanol and determined photometrically This method was fairly accurate giving duplicates with le ations of l ss than ±3 to 5 per cent and allowing quantitative deter mination of as little as 1 5 μg of spermine Inconveniently the relationship between ninhydrin colour and amine concentration was not l near Consequently

SHAW 1947) This method was modified by ROSENTHAL and TABOR (1956) for quantitative estimation of spermidine and spermine using *tertiary* butanol for extraction With this procedure about 90 per cent of the polyamines were recovered from the butanol phase Steam distillation has also been used as a preliminary to the subsequent separation of amines by paper electrophoresis (HERBST, WEAVER and KEISTER 1958)

For a further purification of the amino fraction and for separation of the amines from each other several methods have been introduced

A paper chromatographic technique has been used by several authors for the qualitative separation of spermidine and spermine (*e g* BREMNER and KENTEN 1951 ACKERMANN 1955 FISCHER and BOHN 1957a HERBST KEISTER and WEAVER 1958 AMES DUBIN and ROSENTHAL 1958) but has also been applied to quantitative analyses (RAZIN and ROZANSKY 1957 RAZIN GERY and BACHRACH 1959)

A paper electrophoretic technique has also been used for the qualitative separation of polyamines (FISCHER and BOHN 1957a HERBST KEISTER and WEAVER 1958) Using citric acid or sulphosalicylic acid buffer systems in paper electrophoresis HERBST KEISTER and WEAVER (1958) obtained a distinct and reproducible separation of the individual components in complex mixtures of monoamines diamines or polyamines The paper electrophoretic technique allowed a rapid separation of several amines occurring in the animal organism *e g* 1 4 diaminobutane (putrescine) spermidine and spermine which was also suitable for quantitative analyses (FISCHER and BOHN 1957b LAHDEVIRTA RAINA and HEIKEL 1957) Quantitative paper electrophoresis has further been used for clarifying the metabolism of diamines and polyamines in micro organisms (WEAVER and HERBST 1958 RAZIN GERY and BACHRACH 1959) High voltage paper electrophoresis was used by ZILLIG KRONE and ALBERS (1959) for analysing the amines from ribosomes

An adequate separation of various diamines and polyamines for quantitative analysis is also possible with ion exchange chromatography According to ROSENTHAL and TABOR (1956) ion exchange resins such as Amberlite XF 64 and Dowex 50 were suitable for chromatography Deproteinized tissue extracts as such or purified by butanol extraction could be placed on the column Elution was carried out with sodium sulphate in phosphate buffer or with hydrochloric acid Under special conditions when only spermine was to be determined as little as 2 μg per sample could be analysed quantitatively However when a complex mixture of various bases had to be analysed several successive chromatographic procedures with different adsorbents were required to achieve a complete separation (TABOR ROSENTHAL and TABOR 1958) Ion exchange chromatography was also used for the separation of the various reaction products in clarifying the biosynthesis and degradation of spermidine and spermine (RAZIN GERY and BACHRACH 1959 DUBIN 1960 TABOR 1962a b)

spermine (expressed as phosphate) per 100 g of fresh tissue Further organs relatively rich in spermine were ox liver kidneys and spleen The highest value however was obtained from human semen namely 260 mg of spermine phosphate per 100 g of semen On the other hand none of this base could be isolated from bull semen Nor was any spermine found in ox blood or hen's eggs

A number of later studies have concerned the occurrence of spermine in human organs (WREDE 1926 HARRISON 1931 1933) According to HARRISON (1931) the spermine in human semen is principally derived from the prostate which was found to contain 130 mg of spermine phosphate per 100 g of tissue HÄMÄLÄINEN (1941) analysed a necropsy series consisting of 69 cadavers The flavianate method used by him also signified an improvement in analytical technique Some average values expressed as mg of phosphate per 100 g wet weight obtained by HÄMÄLÄINEN were as follows prostate 122 9 pancreas 28 0 liver 14 3 spleen 11 0 adrenals 9 4 and kidneys 7 7 All in all spermine could be isolated from twenty different tissues In some cases spermine was also found in blood In general marked differences in spermine content between the same organs from different cadavers were observed and no clear correlation with any particular disease was observed However in leukemia a high content was found in the bone marrow and liver

The introduction of more sensitive analytical methods made it possible to study the occurrence of spermidine and spermine in small laboratory animals ROSENTHAL and TABOR (1956) who analysed a number of organs of various laboratory animals generally obtained higher contents than those presented above Thus rat pancreas was shown to contain 194 µg of spermine and 1950 µg of spermidine expressed as free base per g of wet weight In mice and rats the content of spermidine was as high as or even higher than that of spermine Another interesting observation was the high content of spermidine in the newborn rat In chicken liver they found only traces of polyamine which was suggested to be due to a high turn over rate

More recently the occurrence of certain diamines viz putrescine (1 4 diaminobutane) and 1 3 diaminopropane has been reported besides the polyamines in some animal tissues (FISCHER and BOHN 1957b WEAVER and HERBST 1958) These findings are of interest because putrescine can act as a precursor in the biosynthesis of polyamines in micro organisms (cf p 15) and 1 3 diaminopropane has been shown to be one of the *degradation* products of polyamines in micro organisms (p 17)

The occurrence of spermine in the blood of man has been reported by ROSENTHAL and TABOR (1956) This was found in the formed elements principally in leucocytes and none in the plasma Recently the occurrence of both spermine and spermidine in normal human blood was established by PAIVA (1962a) In all cases studied small amounts of these amines were found in the cell fraction but none in the plasma

A number of studies have purported to demonstrate the presence of spermine in the serum in certain pathological conditions for example in malignant diseases (e g MOOGIAN 1953 for further references see TABOR TABOR and ROSENTHAL 1961) In most cases a colour reaction obtained by treating the sample with copper carbonate has been used However this method has been

a standard with a concentration near to that of the unknown sample had to be run simultaneously

Another ninhydrin method introduced by *GIRI RADHAKRISHNAN and VAIDYANATHAN* (1952) for the paper chromatographic study of transamination reactions has also been used by some authors for quantitative estimation of amines after paper chromatographic separation (RAZIN and ROZANSKY 1957 RAZIN GERY and BACHRACH 1959 *BACHRACH PERSKY and RAZIN 1960*) In principle this method is the same as that described above with the difference that the coloured ninhydrin spots were eluted with ethanol solution containing copper sulphate However no information about the accuracy or sensitivity is available With the method used by *KAY HARRIS and ENTENMAN (1956) for* quantitative estimation of amino acids on paper WEAVER and HERBST (1958) could detect as little as 0 01 μmole of amine after paper electrophoretic separation In addition absorption was linear up to 1 5 μmole In this modification paper *strips were sprayed with alkaline ninhydrin solution without addition of* copper salt ZILLIG KRONE and ALBERS (1959) analysed the ribosomal di and polyamines quantitatively with ninhydrin reagent prepared according to MOORE and STEIN (1954) Their method consisted of elution of polyamines *separated by high voltage electrophoresis after which the amines were heated* in ninhydrin solution and determined photometrically

Diazotized *p* nitroaniline was used for the determination of some di and polyamines after paper electrophoretic separation (LAHDEVIRTA RAINA and *HEIKEL 1957) However absorption was not linear and in addition a* strict standardization of the procedure was required when this reagent was used Deviations of duplicates were less than ± 5 per cent of the mean for spermine and spermidine

Microbiological methods based on the growth factor activity of some di and polyamines have also been devised Thus *Haemophilus parainfluenzae* which requires putrescine for growth has been used as a test organism (HERBST WEAVER and KEISTER 1958) Conversely inhibition of the growth of a sensitive *strain of Staphylococcus aureus in the presence of spermine* has been used for the quantitative determination of this amine (RAZIN and ROZANSKY 1959)

OCCURRENCE AND METABOLISM OF SPERMIDINE AND SPERMINE

OCCURRENCE

A number of studies regarding the occurrence of spermidine and spermine in animal tissues and micro organisms have been reported The values obtained with the older gravimetric methods are generally low but nevertheless provide a certain standard of comparison Some of the results are cited below

Animal tissues The wide distribution of spermine in various mammalian organs was demonstrated long ago (DUDLEY and ROSENHEIM 1925) According to these investigators ox pancreas for example contained 25 to 30 mg of

of the high affinity of these basic substances for nucleic acids and phospholipids (see p 18) Secondary redistribution of polyamines during and after homogenization must therefore be taken into account

It was reported by ZILLIG KRONE and ALBERS (1959) that they have demonstrated the presence of 1 3 diaminopropane putrescine and cadaverine (1 5 diaminopentane) in *E coli* ribosomes In liver ribonucleoprotein particles they found putrescine cadaverine and spermidine Quantitative analysis of *E coli* particles indicated that diamines were present in sufficient amounts to neutralize at least a third of the acidic groups of the ribonucleic acids However as pointed out by TABOR TABOR and ROSENTHAL (1961) a secondary redistribution was not excluded COHEN and LICHTENSTEIN (1960) showed that of the total amount of putrescine and spermidine isolated from *E coli* 1° to 15 per cent was in the ribosomal fraction According to these authors the total molar amount of these amines was only 5 to 8 per cent of the molar amount of the ribosomal ribonucleic acid phosphorus Experiments with labelled polyamines showed that the polyamines were attached to the ribosomes prior to cell disruption and that the polyamines were not adsorbed onto the ribosomes in significant amounts after formation of the extract Recently SPAHR (1962) has likewise isolated several bases from *E coli* ribosomes including putrescine spermidine and cadaverine in the molar ratio 3 7 1 The total content of these amines amounted to 0 4 per cent (wt /wt) of the dry ribosomes

BACHRACH and COHEN (1961) studied the localization of spermidine in cells of *Bacillus subtilis* The ^{14}C spermidine formed by the growing cells was localized in both the protoplasts and the bacterial cell walls TABOR (1962c) investigated the binding of labelled spermine by cells and protoplasts of *E coli* As possible binding sites of polyamines diaminopimelic acid in the mucopeptide layer of the inner cell wall or phosphate groups in the outer layers of the cell wall were considered

BIOSYNTHESIS

The biosynthesis of spermidine and spermine has been little understood until the last few years The studies cited below have thrown considerable light on the mechanism of the process in micro organisms On the other hand very little has been reported about the synthesis of these amines in animal tissues A small incorporation of labelled putrescine into the polyamine fraction has been observed however in minced rat prostate No incorporation was found in minced liver muscle spleen or kidney (TABOR ROSENTHAL and TABOR 1956)

Studies with radioactive putrescine labelled with ^{15}N and ^{14}C demonstrated that this substance was incorporated as a unit into polyamines in growing cultures of *E coli* and *Aspergillus nidulans* (TABOR ROSENTHAL and TABOR 1956) being the source of the four carbon chain of polyamines Then it was reported by GREENE (1957) that in *Neurospora crassa* cultures DL methionine ^{14}C was incorporated into spermidine By oxidizing the resulting radioactive spermidine with permanganate it was further shown that methionine is the source of the three carbon chain of spermidine It was also demonstrated that

claimed to be insensitive and not very specific for spermine (GROPIER WITTIG and GRIMALT 1957 ZEITLER 1961) and the results require to be confirmed by other methods Further spermine was found as a component in a new phospholipid »malignolipin» (KOSAKI *et al* 1958) which could be isolated from human malignant tumours but not from normal tissues KAMAT (1962) however using the same technique as reported by KOSAKI *et al* could not obtain a compound like »malignolipin» from five different types of malignant tumours

The occurrence of polyamines in free or bound form has also been demonstrated in some invertebrates (ACKERMANN 1952 ACKERMANN and JANKA 1954 FISCHER and BOHN 1957a)

Micro organisms and viruses The occurrence of spermine in several yeasts was already reported by DUDLEY and ROSENHEIM (1925) HERBST and SNELL (1949) observed that putrescine was an essential growth factor for *Haemophilus parainfluenzae* and could be replaced by spermidine or spermine One or several of these growth factors could be found in a variety of micro organisms (HERBST WEAVER and KEISTER 1958 WEAVER and HERBST 1958) Recently BACHRACH and COHEN (1961) have analysed several micro organisms for their spermidine content The highest value 21 μg of spermidine per mg of protein was obtained in extracts of *Neurospora crassa*

The amine content is markedly affected by the pH of the culture medium (TABOR ROSENTHAL and TABOR 1958) More spermidine relative to putrescine could be isolated from *Escherichia coli* cells grown in an alkaline medium than from cells grown in an acid one

Some micro organisms e g *E coli* and *Staphylococcus aureus* are able to acetylate putrescine and polyamines (DUBIN and ROSENTHAL 1960 ROSENTHAL and DUBIN 1962) Conjugates between glutathione and spermidine or spermine have also been isolated from *E coli* cells (DUBIN 1959) There is no evidence to suggest that similar polyamine derivatives occur in mammalian tissues

Great interest attaches to observations that certain viruses contain diamines and polyamines It has been reported by AMES DUBIN and ROSENTHAL (1958) that putrescine and spermidine occur in phage T4 of *E coli* B in quantities even sufficient for neutralization of one third to one half of the deoxyribonucleic phosphate Spermine was found in the *Salmonella* phage PTL 22 The amines were injected together with deoxyribonucleic acid into the host cells Moreover in experiments with radioactive putrescine direct exchange of the putrescine in the medium with the phage putrescine was ruled out Phage 3 of *E coli* was shown to contain spermidine and putrescine (KAY 1959) More recently AMES and DUBIN (1960) have studied a number of other viruses Certain *E coli* and *Salmonella typhimurium* phages did not contain polyamines It was suggested by these authors that this difference is linked with the greater permeability of these viruses so that during the isolation of the phage displacement of polyamines by the Mg^{++} in the suspending medium could occur Further evidence of the general occurrence of diamines and polyamines in phages has been presented by KAY and FILDES (1962)

Intracellular distribution The question of the intracellular distribution of the polyamines is interesting but its elucidation presents certain difficulties because

of the high affinity of these basic substances for nucleic acids and phospholipid (see p. 18). Secondary redistribution of polyamines during and after homogenization must therefore be taken into account.

It was reported by ZILLIG, KRONE and ALBERS (1959) that they have demonstrated the presence of 1,3-diaminopropane, putrescine and cadaverine (1,5-diaminopentane) in *E. coli* ribosomes. In liver ribonucleoprotein particles they found putrescine, cadaverine and spermidine. Quantitative analysis of *E. coli* particles indicated that diamines were present in sufficient amounts to neutralize at least a third of the acidic groups of the ribonucleic acids. However, as pointed out by TABOR, TABOR and ROSENTHAL (1961), a secondary redistribution was not excluded. COHEN and LICHTENSTEIN (1960) showed that of the total amount of putrescine and spermidine isolated from *E. coli* 12 to 15 per cent was in the ribosomal fraction. According to these authors, the total molar amount of these amines was only 5 to 8 per cent of the molar amount of the ribosomal ribonucleic acid phosphorus. Experiments with labelled polyamines showed that the polyamines were attached to the ribosomes prior to cell disruption and that the polyamines were not adsorbed onto the ribosomes in significant amounts after formation of the extract. Recently SPAHR (196_) has likewise isolated several bases from *E. coli* ribosomes, including putrescine, spermidine and cadaverine, in the molar ratio 3 : 9 : 1. The total content of these amines amounted to 0.4 per cent (wt./wt.) of the dry ribosomes.

BACHRACH and COHEN (1961) studied the localization of spermidine in cells of *Bacillus subtilis*. The ^{14}C-spermidine formed by the growing cells was localized in both the protoplasts and the bacterial cell wall. TABOR (1962c) investigated the binding of labelled spermine by cells and protoplasts of *E. coli*. As possible binding sites of polyamines, diaminopimelic acid in the mucopeptide layer of the inner cell wall or phosphate groups in the outer layers of the cell wall were considered.

BIOSYNTHESIS

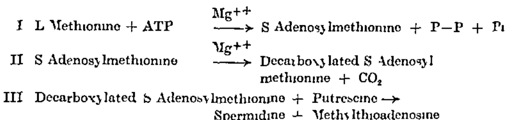
The biosynthesis of spermidine and spermine has been little understood until the last few years. The studies cited below have thrown considerable light on the mechanism of the process in micro-organisms. On the other hand, very little has been reported about the synthesis of these amines in animal tissues. A small incorporation of labelled putrescine into the polyamine fraction has been observed, however, in minced rat prostate. No incorporation was found in minced liver, muscle, spleen or kidney (TABOR, ROSENTHAL and TABOR 1956).

Studies with radioactive putrescine labelled with ^{15}N and ^{14}C demonstrated that this substance was incorporated as a unit into polyamines in growing cultures of *E. coli* and *Aspergillus nidulans* (TABOR, ROSENTHAL and TABOR 1956), being the source of the four-carbon chain of polyamines. Then it was reported by GREENE (19_) that in *Neurospora crassa* cultures DL-methionine-^{14}C was incorporated into spermidine. By oxidizing the resulting radioactive spermidine with permanganate it was further shown that methionine is the source of the three-carbon chain of spermidine. It was also demonstrated that

adenosine triphosphate was required for the incorporation of methionine by cell free extracts of *Neurospora crassa*

These results have been confirmed with cell free extracts and partially purified enzymes from *E coli* (TABOR ROSENTHAL and TABOR 1957 1958) Synthesis of spermidine from putrescine required L methionine adenosine triphosphate and Mg++ L methionine and adenosine triphosphate could be substituted by adenosyl L methionine Radioactivity was not incorporated when methionine labelled in the methyl or carboxyl group was used It was further stated by these authors that 2 ^{14}C labelled ornithine could also be incorporated into spermidine When ^{14}C labelled putrescine was added to the *growth medium of Saccharomyces cerevisiae* both labelled spermidine and spermine were recovered

Purification of the enzymes catalysing the various steps in the biosynthesis of spermidine from putrescine and methionine in *E coli* has been reported (TABOR and TABOR 1960 TABOR 1962a b) According to these authors the enzymic synthesis of spermidine involves the following three steps



I L Methionine + ATP $\xrightarrow{Mg^{++}}$ S Adenosylmethionine + P—P + Pi

II S Adenosylmethionine $\xrightarrow{Mg^{++}}$ Decarboxylated S Adenosyl methionine + CO_2

III Decarboxylated S Adenosylmethionine + Putrescine → Spermidine + Methylthioadenosine

The enzyme that catalyses the formation of S adenosylmethionine is similar to the methionine activating enzyme isolated from liver by CANTONI and DURELL (1957) The *E coli* enzyme has been purified 1500 fold The enzyme *that catalyses the decarboxylation of S adenosylmethionine has been purified* twentyfold This enzyme which requires Mg++ as activator was inhibited by sodium cyanide No effect on activity was exerted by pyridoxal or pyridoxamine phosphate The enzyme that catalyses the third step also called propylamine transferase, has been purified a thousandfold from *E coli* For the purified enzyme no cofactor requirements have been observed Putrescine but not spermidine could act as an acceptor of the propylamine moiety The enzymic synthesis of spermine has not yet been demonstrated

That methionine can act as precursor of spermidine has also been demonstrated recently by *BACHRACH and COHEN (1961)* with several micro organisms 1 3 Diaminopropane has also been stated to act as a precursor of spermidine (WEAVER and HERBST 1958) but this finding was not confirmed with isotope methods

DEGRADATION

Mammals HIRSCH (1953a b) observed that beef and sheep sera contained an enzyme which attacked spermidine and spermine causing a rapid oxidative *deamination of these amines* which was reflected by consumption of oxygen

and liberation of ammonia Carbonyl reagents almost completely blocked the action of this enzyme Histamine tyramine and various aliphatic diamines were not attacked *On account of its substrate specificity the name* spermine oxidase was proposed by HIRSCH Sera obtained from man rabbit guinea pig horse and pig were inactive against polyamines

This soluble beef plasma enzyme has been purified 150 to 200 fold by TABOR TABOR and ROSENTHAL (1954) In addition to polyamines several aliphatic amines *and benzylamine were oxidized* but not tryptamine 5 hydroxy tryptamine or epinephrine Spermidine could be identified as an intermediate in the oxidation of spermine The formation of spermidine from spermine by sheep serum has also been observed by BACHRACH and BAR OR (1960) An unidentified aldehyde and putrescine are found as other degradation products (TABOR and ROSENTHAL *1956) The exact nature* and stoichiometry of the oxidation of polyamines are not known at present

Little is yet known concerning the chemical nature of plasma spermine oxidase According to HIRSCH (1953a) its mobility in electrophoresis was like that of alpha globulin Inhibition by carbonyl reagents suggests the presence of a carbonyl *group in the active centre* (BLASCHKO 1962) Recently it has been reported by GORKIN (1961) that zinc participates in spermine oxidase activity The absorption spectrum of the purified enzyme did not contain bands characteristic of vitamin B_6 or B_2 derivatives

The occurrence of spermine oxidase in the sera of all ruminants but not *of non ruminants* (BLASCHKO *and* HAWES 1958 1959) *is a peculiar feature It* has been further shown that spermine oxidase activity is low or absent in goat serum immediately after birth but tends to increase gradually with time The ontogenetic course of development of spermine oxidase its relation to other amine oxidases its distribution and functional significance have recently been discussed by BLASCHKO (1962) *in an extensive review to which the reader is* referred

Very little is known of the metabolic fate of polyamines in animal tissues Attempts to show degradation of polyamines *in vitro* have been virtually unsuccessful except for experiments with crude preparations from hog kidney which showed some activity (ZELLER 1938) *and those reported by* HIRSCH (1953a) who found tuberculostatic activity in guinea pig kidney and heart homogenates when incubated with spermine In contrast TABOR and ROSENTHAL (1956) could not demonstrate degradation of spermine in minced tissues or homogenates from liver spleen and lung But spermine was degraded *in vivo* (ROSENTHAL and TABOR 1956) When *spermine was administered to mice* rats and rabbits a small part appeared in the urine as spermidine Apart from the report of ZELLER cited above nothing is known about the fate of spermidine either *in vivo or in vitro*

Micro-organisms It has been shown that a number of micro organisms are able to oxidize polyamines Several *degradation products have been identi*fied SILVERMAN and EVANS (1944) found that spermine and spermidine were rapidly oxidized by *Pseudomonas aeruginosa* Recent studies have shown that this *organism oxidizes* spermine to spermidine which is further degraded to 1 3 diaminopropane β alanine and γ aminobutyric acid Aldehydes were

detected as intermediates 1 3 Diaminopropane was oxidized to β alanine Degradation was inhibited by carbonyl reagents such as hydroxylamine and semicarbazide (RAZIN BACHRACH and GERY 1958 RAZIN GERY and BACHRACH 1959) Several other micro organisms e g *Mycobacterium smegmatis* (BACHRACH PERSKY and RAZIN 1960) are able to oxidize polyamines Some differences have been found in substrate specificity and reaction products These are described in detail in the review of TABOR TABOR and ROSENTHAL (1961) to which the reader is referred

Recently an enzyme from spermidine adapted cells of *Serratia marcescens* has been purified 20 to 30 fold by BACHRACH (1962a b) This enzyme oxidized spermidine but did not attack spermine short chain diamines or benzylamine 1 3 Diaminopropane and pyrroline were found as oxidation products of spermidine in stoichiometric amounts γ aminobutyraldehyde was suggested as an intermediate which would spontaneously form pyrroline Ammonia was not liberated As pointed out by BACHRACH this spermidine oxidase from *Serratia marcescens* differed from the plasma spermine oxidase described both in specificity and in reaction products

POSSIBLE PHYSIOLOGICAL ROLL OF POLYAMINES

Although the physiological role of polyamines is at present obscure recent studies on these substances have afforded much additional data which should eventually lead to an understanding of their function Various stabilizing effects of polyamines on certain bacteria protoplasts and bacterial cell walls have been shown in a number of studies Polyamines also exert a stabilizing effect on preparations of mitochondria Reference may be made to the recent review by TABOR TABOR and ROSENTHAL (1961) where the above mentioned effects and their possible mechanisms are described and discussed in detail

Complex formation of polyamines One possible mechanism of the stabilization and protection by polyamines could be explained by the high affinity of these compounds for cell constituents having acidic groups In a number of studies it has been demonstrated that polyamines are able to form complexes with nucleic acids (KEISTER 1958a b RAZIN and ROZANSKY 1959 CANTONI 1960 FELSENFELD and HUANG 1960 AMES and DUBIN 1960) with lecithin (RAZIN and ROZANSKY 1959) and with heparin (AMANN and WERLE 1956) According to KEISTER (1958b) the complexes of polyamine with nucleic acids are stable within a large pH range but are completely dissociated by 1 5 to 2 per cent sodium chloride The reversibility of complexing by inorganic salt was also found by RAZIN and ROZANSKY (1959) CANTONI (1960) found differences in solubilities of complexes between spermine and soluble ribonucleic acids from rabbit liver Of the synthetic polyribonucleotides polyadenylic and polyinosinic acids were precipitated by equivalents of spermine per mol of polymer phosphorus whereas polycytidylic and polyuridylic acids were precipitated only with excess of spermine (HUANG and FELSENFELD 1960) It was further shown by the same authors (FELSENFELD and HUANG 1960) by means of

conductometric titrations that spermine was capable of displacing barium stoichiometrically from polyuridylic acid which was precipitated as a spermine polyuridylic acid complex It was suggested that in physiological situations nucleic acids carry significant amounts of polyamines The quantity of polyamines carried would depend on the concentration of small ions which can compete for sites on the nucleic acids

Stabilization of nucleic acids by polyamines The protective effect of polyamines and to a lesser extent of diamines against denaturation and enzymic degradation is obviously due to complex formation According to KEISTER (1958b) spermine at a concentration of 2.5×10^{-4}M completely blocked the degradation of both ribo and deoxyribonucleic acids in crude bacterial sonicates Spermine inhibited purified ribonuclease and deoxyribonuclease although not completely The inhibiting action of spermine on the autolytic degradation of bacterial ribonucleic acid was also noted by HERBST and DOCTOR (1959) The stabilizing effect of polyamines on deoxyribonucleic acid has recently been confirmed (MAHLER MEHROTRA and SHARP 1961 TABOR 1962) Spermine and spermidine protected deoxyribonucleic acid from calf thymus and from *Bacillus subtilis* against heat denaturation spermine even in concentrations of 10^{-6} to 10^{-5}M if the salt concentration in the medium was relatively low The protective effect was manifested as an increase of the melting temperature Larger amounts of these amines were required to demonstrate the same effect in the presence of higher ionic strengths (TABOR 1962) Similarly spermine stabilized a transforming deoxyribonucleic acid from *Bacillus subtilis* against heat inactivation (TABOR 1961)

Stabilization of bacteriophages by polyamines The stabilizing effects on bacteriophages are also apparently due to complex formation between polyamines and nucleic acid (FRASER and MAHLER 1958 TABOR 1960 FRASER and MAHLER 1961 MORA YOUNG and RIZVI 1962) According to TABOR (1960) even in concentrations of 10^{-4} to 10^{-7}M spermine and spermidine were effective in stabilizing *E coli* bacteriophage T5 This effect was most pronounced at low salt concentrations

AMES and DUBIN (1960) studied the role of di and polyamines in bacteriophage They stated that the normal cation content of phage T4 of *E coli* which was in equilibrium with the total deoxyribonucleic acid anions consisting of putrescine spermidine and Mg^{++} was changed under certain conditions When the host cell were grown in a medium containing spermine this polyamine and its acetylated derivatives replaced the normal constituents spermidine and putrescine Thus the phage polyamines were qualitatively determined by the polyamine content of the host cells It was concluded that the role of the polyamines in phages is probably that of a non specific cation for neutralization and stabilization of deoxyribonucleic acid This was supported by the finding that in a mutant of phage T4 all cations neutralizing deoxyribonucleic acid could be displaced without loss of infectivity

Stabilizing effect on ribosomes Some recent studies have demonstrated the stabilizing effect of polyamines and diamines on ribosomes (COHEN and LICHTENSTEIN 1960 COLBOURN WITHERSPOON and HERBST 1961 COHEN and BARNER 196) COHEN and LICHTENSTEIN (1960) observed that spermidine

detected as intermediates 1,3-Diaminopropane was oxidized to β-alanine. Degradation was inhibited by carbonyl reagents such as hydroxylamine and semicarbazide (RAZIN, BACHRACH and GERY 1958; RAZIN, GERY and BACHRACH 1959). Several other micro-organisms, e.g. *Mycobacterium smegmatis* (BACHRACH, PERSKY and RAZIN 1960) are able to oxidize polyamines. Some differences have been found in substrate specificity and reaction products. These are described in detail in the review of TABOR, TABOR and ROSENTHAL (1961) to which the reader is referred.

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Although the physiological role of polyamines is at present obscure, recent studies on these substances have afforded much additional data which should eventually lead to an understanding of their function. Various stabilizing effects of polyamines on certain bacteria, protoplasts and bacterial cell walls have been shown in a number of studies. Polyamines also exert a stabilizing effect on preparations of mitochondria. Reference may be made to the recent review by TABOR, TABOR and ROSENTHAL (1961) where the above-mentioned effects and their possible mechanisms are described and discussed in detail.

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incorporation in the presence of polyuridylic acid in a system containing *Salmonella typhimurium* ribosomes (MARTIN and AMES 196) In the e experiments also the stimulating effect was most pronounced at suboptimal levels of Mg^{++}

The mode of action of polyamines in the amino acid incorporating system requires further elucidation The parallelism between these effects of polyamines and Mg^{++} which was mentioned above (HERSHKO AMOZ and MAGER 1961) has been confirmed in another work (MAGER BENEDICT and ARTMAN 1962) It has been suggested that these effects are due to stabilization of the ribonucleoprotein particles (HERSHKO AMOZ and MAGER 1961) MARTIN and AMES (1962) pointed out the ability of polyamines to convert nonfunctional 70 S ribosomal particles to functional 100 S particles

Some recent reports indicate that polyamines are able to affect nucleotide synthesis KEISTER (1958a) observed that spermine stimulated nucleic acid synthesis in the pre ence of chloramphenicol A striking effect of spermine on polyribonucleotide synthesis in the *E coli* membrane fraction has been reported by SPIEGELMAN (1959) In the absence of spermine the polyribonucleotides synthesized tended afterwards to degrade This degradation was inhibited by spermine Polyamines were also capable of stimulating the incorporation of adenine nu leoti ls into ribonucleic acid (CHUNG and MAHLER 1959)

but not putrescine was able to preserve *E. coli* ribosomal constituents which otherwise tended to decompose. Mg^{++} had the same effect. Mg^{++} and spermidine together had an even greater effect. On the basis of their experiments the authors suggested that Mg^{++} and spermidine were the naturally occurring clasps on the complex ribosomal components in the bacteria. COLBOURN, WITHERSPOON and HERBST (1961) found that polyamines increased the size of *E. coli* ribosomes. When the cells were grown in a glucose salt medium or in the same medium with added spermine, a complete absence of larger ribosomes was observed in the former case, whereas in the latter experiment large ribosomes (96.6 S and 65.5 S) were also observed. Spermine was incorporated into ribosomes during the exponential growth of bacteria. It was considered that the role of spermine is similar to Mg^{++} in the formation and preservation of large ribosomes.

Recently SIEKEVITZ (1961) has described the effects of spermine on ribosomes from guinea pig pancreas. On removal of Mg^{++} from the particle with chelating agents, bound amylase and ribonuclease as well as most of the ribosomal ribonucleic acid were also removed from the particles. It was found that the bound enzymes were released by diamines and even more readily by spermine. Spermine replaced the ribosomal Mg^{++} and stabilized the particles in the absence of Mg^{++} by inhibiting the leakage of ribonucleic acid almost completely.

Effects on amino acid incorporation and nucleotide synthesis. Diamines and polyamines are able to stimulate *in vitro* incorporation of amino acids into ribonucleoprotein particles (HERSHKO, AMOZ and MAGER 1961; TISSIÈRES and HOPKINS 1961; DOERFLER *et al.* 1962; MAGER, BENEDICT and ARTMAN 1962; MARTIN and AMES 1962; LUCAS, SCHUURS and SIMPSON 1963). Inhibition by spermine of haemoglobin synthesis in reticulocytes and cell free systems has also been reported (ALLEN and SCHWEET 1962). According to YOSHIKAWA and MARUO (1960), polyamines and diamines are able to stimulate amylase formation.

HERSHKO, AMOZ and MAGER (1961) showed that spermine in a concentration of 10^{-4}M stimulated the incorporation of labelled leucine into rat liver microsomes and ribosomes. This effect was most pronounced at suboptimal levels of Mg^{++}, the amount incorporated being above the maximum value reached with optimal Mg^{++} concentration alone. When the Mg^{++} content was optimal (about 10^{-2}M) the spermine effect became inhibitory. The latter effect was considered to be due to a shift of the Mg^{++} excess inhibition zone to lower levels of Mg^{++}. Evidence was presented in support of the hypothesis that the site at which the spermine was acting is located in the ribonucleoprotein particle. Spermidine, putrescine and agmatine had effects similar to spermine, although less marked.

Spermidine and spermine also had a stimulating effect on the incorporation of amino acids by *E. coli* ribosomes (TISSIÈRES and HOPKINS 1961; DOERFLER *et al.* 1962). Addition of deoxyribonucleic acid to the reaction mixture increased the amino acid incorporation. When deoxyribonucleic acid was added together with small amounts of spermidine, this increase was even higher, whereas spermidine alone had no significant effect in this system (TISSIÈRES and HOPKINS 1961). Polyamines and diamines also markedly increased phenylalanine

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Spermidine and spermine also had a stimulating effect on the incorporation of amino acids by *E coli* ribosomes *(TISSIÈRES and* HOPKINS 1961 DOERFLER *et al* 1962) Addition of deoxyribonucleic acid to the reaction mixture increased the amino acid incorporation When deoxyribonucleic acid was added together with small amounts of spermidine this increase was *even higher whereas* spermidine alone had no significant effect in this system (TISSIÈRES and HOPKINS 1961) Polyamines and diamines also markedly increased phenylalanine

reagent for accurate work. Many attempts were made to develop a method which would permit an accurate and if possible specific determination of spermidine and spermine directly on paper without previous elution of these amines. As only a rather small amount of amine can be applied to paper, a high sensitivity is required. Neither spraying the paper strips with dinitrofluorobenzene reagent (ISHERWOOD and CRUICKSHANK 1954) nor colorimetric estimation (ROSENTHAL and TABOR 1956) after elution of spermidine and spermine from the paper gave satisfactory results. Dyeing with flavianic acid by spraying the paper strips with an aqueous solution of flavianic acid was also tried, but spermine was precipitated on the paper only when present in large amounts and spermidine not at all.

Selection of amido black as quantitative reagent for polyamines. In connexion with paper electrophoretic fractionation of human seminal plasma, which contains large amounts of spermine, some authors have found a basic fraction which moves rapidly to the cathode and forms a poorly soluble complex with acidic protein dyes. Thus CAMBA and CARTA (1954) observed a fraction moving rapidly to the cathode, which stained with bromphenol blue. They identified this fraction as spermine. SCHNEIDER, NOWAKOWSKI and VOIGT (1954) found a similar fraction which stained bluish violet with amido black in contrast to protein. They considered this fraction to be mucous matter. In the secretions of bird spiders the basic complex compounds, which also took up amido black, contained 1,3-diaminopropane and spermine (FISCHER and BOHN 1957a). Intensive staining of the spermine fraction with amido black was also noted by LUUKKAINEN (1957, personal communication) in a paper electrophoretic study of spermatic fluid. This observation led the present author to try to devise a quantitative method using amido black or some other protein dye as reagent. More recently, staining of spermine with amido black has also been observed by ESTBORN and SWEDIN (1959).

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PRESENT INVESTIGATION

DETERMINATION OF SPERMIDINE AND SPERMINE

MATERIAL

Reference standards Spermidine phosphate and spermine tetra hydrochloride were used as reference standards in the present study These were preparations obtained from F Hoffmann — La Roche & Co Basel or from Mann Research Laboratories Inc New York The standards usually prepared in 0 1 N hydrochloric acid were stable for several months at + 3°C

Reagents The amido black used as quantitative reagent for polyamines was obtained from E Merck Darmstadt (Amidoschwarz 10 B fur Elektrophorese und Redoxindikator) Reagents used in preliminary experiments were 2 4 dinitro 1 fluorobenzene (Hoffmann — La Roche) flavianic acid (Hoffmann — La Roche) Azocarmine B (Farbenfabriken Bayer AG Leverkusen) bromphenol blue (Merck) and lissamine green (British Drug Houses [B D H] Ltd London) The anhydrous sodium sulphate and trisodium phosphate were manufactured by May & Baker Ltd Dagenham The sulphosalicylic acid used as buffer was obtained from Merck

Solvents *n* butanol (B D H) ethylene glycol monomethyl ether (Merck) propionic acid (Fluka AG Buchs SG) and pyridine (Baker Chemical Co Phillipsburg Missouri) Methanol used for washing both *p a preparation from Merck and technical methanol containing* 99 8 weight per cent methanol (Enso Gutzeit Oy Imatra) were used The technical methanol was redistilled at 64—65°C before use

Other reagents used were *p a* preparations obtained from Merck

PRELIMINARY EXPERIMENTS

As seen from the review of the literature the chemical methods used for the determination of polyamines are in no way specific In addition, *p* nitroaniline was found to be too labile and insensitive a

reagent for accurate work. Many attempts were made to develop a method which would permit an accurate and if possible specific determination of spermidine and spermine directly on paper without previous elution of these amines. As only a rather small amount of amine can be applied to paper a high sensitivity is required. Neither spraying the paper strips with dinitrofluorobenzene reagent (ISHERWOOD and CRUICKSHANK 1954) nor colorimetric estimation (ROSENTHAL and TABOR 1956) after elution of spermidine and spermine from the paper gave satisfactory results. Dyeing with flavianic acid by spraying the paper strips with an aqueous solution of flavianic acid was also tried but spermine was precipitated on the paper only when present in large amounts and spermidine not at all.

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molecule and a mol wt of 572 (DITTMER 1961, p 78) It stains very intensely thus being useful for the detection of minute amounts of polyamines

Various factors affecting the isolation and paper electrophoretic separation, as well as photometric determination, of spermidine and spermine with amido black are discussed at greater length in the following sections For purposes of orientation, a brief outline of the present method is given below

Tissue is homogenized in dilute hydrochloric acid The homogenate *is deproteinized with trichloroacetic acid The protein free filtrate is* then extracted with ether to remove most of the trichloroacetic acid and made alkaline After addition of a sodium sulphate—*tertiary* sodium phosphate salt mixture the amines are extracted into *n* butanol After extraction the butanol phase is separated, made acidic *and evaporated to dryness The residue is dissolved with dilute* hydrochloric acid and subjected to paper electrophoresis After electrophoresis the paper *strips are dried and stained with amido* black solution, dried again and the extra colour washed off in a methanol—acetic acid bath The coloured amine fractions are cut off eluted with sodium hydroxide and determined photometrically

TISSUE HOMOGENIZATION

Homogenization Technique Effect of Hydrolysis

As yet it has not been demonstrated whether the polyamines *occurring in mammalian tissues are primarily in a firmly* bound form (cf FISCHER and BOHN 1957b) However as spermidine and spermine being highly basic substances have a high affinity for compounds containing acidic groups (see p 18) it seemed possible that during the homogenization procedure a secondary distribution and binding might occur especially if the homogenization medium was nearly neutral This was supported by the finding that in preliminary exper iments when 70 per cent eth*yl alcohol was used for protein precipi* tation instead of trichloroacetic acid very poor recoveries were obtained

In the following experiments one rat liver was cut into slices and divided into two equal portions one of which was homogenized in 0 9 per cent sodium chloride and the other in 0 1 N hydrochloric acid one part of tissue to 4 parts of solution (wt /vol) with an Ultra Turrax homogenizer (Janke & Kunkel KG Staufen) For each homogenate three different procedures were applied In the first series the protein was precipitated with 10 per cent trichloroacetic acid immediately after homogenization *in the second the homogenate* was stored for 24 hours at —18 C and then deproteinized The third series was stored as described above then hydrolysed in 1 N hydro chloric acid for 4 hours at 100 C and finally deproteinized The protein free filtrates were analysed as described on p 43 The results are summarized in Table 1 When 0 1 N HCl was used as homogenization

Table 1 Effect of homogenization technique and hydrolysis on the recovery of spermidine and spermine from rat liver Polyamine content expressed as µg g fresh tissue

Procedure	Homogenization medium			
	0 9 per cent NaCl		0 1 N HCl	
	Spermidine	Spermine	Spermidine	Spermine
I Precipitation immediately after homogenization	99 9 10 9 105 3	183 0 185 9 18 5	1 00 118 9 1 38	199 4 194 6 198 8
Mean	103 7	183 6	1 00	197 6
II Homogenate stored 4 h at – 18 C and then deproteinized	95 6 91 8 95 0	1 3 17 5 1 83	1 49 1?1 1 1 65	199 4 196 ? 199 4
Mean	94 1	1 80	1 4	198 3
III Homogenate stored as in II then hydrolysed in 1 N HCl 4 h at 100 C	104 8 109 1 110 8	196 7 199 9 19 8	1 11 1 38 1 65	199 4 199 9 ?0 5
Mean	108	198 1	1 38	?00 6

medium all three procedures gave practically the same mean recovery that is for spermidine 120 9 124 2 and 123 8 µg/g wet weight and for spermine 197 6 198 3 and 200 6 µg/g wet weight In this case acid hydrolysis did not enhance the recovery In contrast smaller

yields were obtained when 0 9 per cent sodium chloride was used as homogenization medium The mean percentage recoveries if compared with the same series homogenized in hydrochloric acid, were about 86, 76 and 87 per cent for spermidine, and 93 89 and 99 per cent for spermine This indicates that spermidine was bound the binding increasing with storage of the homogenate, acid hydrolysis could *only partly release the bound amine* Spermine seemed to be bound to a smaller extent

Hydrolysis in 3 or 6 N hydrochloric acid at 100°C did *not enhance* the recovery from tissues when 0 1 N HCl was used as homogenization medium When even stronger hydrolysis was tried viz in 6 N HCl at 130°C for 4 hours, the recoveries from the tissues were only 60 to 70 per cent, a yield of 100 per cent was obtained, however, from pure polyamine solutions

Thus it appears that when homogenization was carried out in a *neutral* medium a secondary binding of polyamines occurred The experiments in which hydrochloric acid was used as homogenization medium *do not support the hypothesis that the polyamines occur* primarily in a firmly bound form in rat liver or in whole chick embryo which was also tested This is in agreement with the results reported by FISCHER and BOHN (1957b) These workers found that alkaline hydrolysis did not improve the recovery of spermidine and spermine from several mammalian tissues for which reason they concluded that in the tissues studied these amines occur in entirely unbound form, *i e as* salts

Recovery of Spermidine and Spermine Added to Homogenate

To discover the extent to which spermidine and spermine could be recovered when added to the homogenate the following experiments were made Chick embryos were homogenized in 4 parts (wt /vol) of 0 9 per cent sodium chloride To 2 5 ml of homogenate 20 or 40 μg of spermidine and spermine were added the protein was precipitated after 10 min with 10 per cent trichloroacetic acid and the polyamines determined from the filtrates The results are presented in Table 2 The mean recoveries of added spermidine were 93 3 and 95 9 per cent and of added spermine 96 9 and 98 4 per cent

Table 2 Recovery of spermidine and spermine added to homogenate Homogenate from chick embryo was prepared by homogenizing one part of tissue in 4 parts (wt/vol) of 0 9 per cent sodium chloride the stated amount of polyamines was added and deproteinized with trichloroacetic acid 10 min later Mean value ± standard deviation of 4 determinations

Addition	Spermidine recovered		Spermine recovered	
	μg	per cent of added	μg	per cent of added
No addition	33 ± 1 3		39 5 ± 1 4	
20 μg	51 9 ± 0 [illegible]	93 3 ± 3 6	58 9 ± 0 8	96 9 ± 3 8
40 μg	16 ± 1 6	9 9 ± 4 0	8 9 ± 1 3	98 4 ± 3 3

PURIFICATION AND SEPARATION OF SPERMIDINE AND SPERMINE

Butanol Extraction

To achieve a distinct separation and to increase specificity on paper electrophoresis a previous purification of the protein free tissue extracts is needed In the present work butanol extraction according to McINTIRE ROTH and SHAW (1947) has been employed The following procedure was used 8 ml of protein free filtrate in a glass stoppered test tube was made alkaline (pH 12—13) with sodium hydroxide 2 g of a salt mixture consisting of 62 5 g anhydrous sodium sulphate and 9 g trisodium phosphate was added The solution was then extracted with 8 ml of *n* butanol with vigorous mechanical shaking for 30 min After centrifugation at low speed the butanol phase was sucked up carefully in a pipette with a rubber bulb transferred to a small evaporation dish evaporated to dryness after acidification and then applied to paper electrophoresis

The recovery of spermidine and spermine in the butanol phase was determined by addition of 25 or 50 μg of each polyamine to 8 ml of water and treating as described above The results are presented in Table 3 The mean percentage recovery for spermidine was *91 8* ± *1 5* and for spermine 95 3 ± 0 6 (standard deviation) ROSENTHAL and TABOR (1956) using *tertiary* butanol for extraction obtained approximately *90 per cent* of spermidine and spermine in the butanol phase Thus in the present study somewhat higher yields were

Table 5 Recovery of spermidine and spermine by butanol extraction The amines were extracted from 8 ml of alkaline aqueous solution treated with 2 g of sodium sulphate — trisodium phosphate salt mixture with 8 ml of n butanol for 30 min

Addition μg	Amine recovered in butanol phase			
	Spermidine		Spermine	
	μg	per cent	μg	per cent
25	22 8	91 2	23 6	94 4
»	23 2	92 8	24 0	96 0
»	22 8	91 2	23 8	95 2
50	45 6	91 2	47 3	94 6
»	47 0	94 0	47 4	94 8
»	44 6	89 2	47 6	95 2
»	45 4	90 8	47 8	95 6
»	46 9	93 8	47 9	95 8
»	46 2	92 4	48 0	96 0
Mean ± S D		91 8 ± 1 5		95 3 ± 0 6

obtained with *n* butanol The extraction recovery was checked several times during the course of the work by adding spermidine and spermine to the protein free filtrate prior to butanol extraction and was found to be about 92 per cent for spermidine and 95 per cent for spermine Therefore the standards were not included in the experiments until the paper electrophoresis stage, and the extraction loss of 8 per cent for spermidine or 5 per cent for spermine was taken into account in the calculation of the polyamine content of the unknown sample

The »amine fraction» obtained by extraction of the protein free tissue extract with *n* butanol was by no means pure but contained traces of amino acids and other ninhydrin positive substances which on paper electrophoresis migrated more slowly than did the polyamines (Fig 5 p 40) Thus in addition to polyamines more than ten ninhydrin positive spots could be detected on thin layer chromatograms from chick embryo extracts However the butanol extract was found to be pure enough to allow an adequate separation of polyamines on paper electrophoresis By contrast in some experiments in which protein free tissue extracts were subjected to paper electrophoresis without previous butanol extraction the polyamine fractions were not separated distinctly enough

Apparatus and materials For paper electrophoresis two non commercial apparatuses were used in which the paper strips were placed horizontally on three glass rods 18 paper strips 3 × 39 cm Whatman No 1 could be placed for a simultaneous run Stabilized power of 300 V was employed All runs were made at room tempera ture No cooling device was used

Table 4 Effect of buffer concentration on the recovery of spermidine and spermine on paper electrophoresis Sulphosalicylic acid buffer pH 3 5 Staining with amido black Each value is a mean of 4 or 5 determinations 4 µg of each polyamine per strip

Molar concentr of buffer	Spermidine		Spermine	
	Absorbance	Change per cent	Absorbance	Change per cent
0 07	0 4 0		0 34	
0 06	0 4 0	± 0	0 341	− 0 3
0 05	0 465	− 1 1	0.333	− 6
0 03	0 451	− 4 0	0 317	− 7 3

Selection of buffers 0 065 M sulphosalicylic acid buffer at pH 3 5 was used unless otherwise stated Variations of pH from 2 5 to 5 0 had no significant effect on the recovery of polyamines In contrast when the buffer concentration was varied losses due to adsorption especially in the spermine fraction were noted when the buffer concentration was less than 0 06 M (Table 4) The adsorption of spermidine was smaller With a buffer concentration higher than 0 07 M the separation of spermidine and spermine from each other was not complete 0 065 M buffer gave a good separation and recovery was still optimal Some typical paper electrophoretic strips stained with amido black are seen in Fig 1

Citric acid buffer was also used for paper electrophoresis mainly in isotope experiments The adsorption effect in the spermine fraction at a low buffer concentration (less than 0 07 M) was similar to that described above With 0 1 M citric acid buffer a distinct separation was obtained and adsorption was negligible Sulphosalicylic acid buffer was preferred for routine work however because of the lower background values after staining with amido black

Table 3 Recovery of spermidine and spermine by butanol extraction The amines were extracted from 8 ml of alkaline aqueous solution treated with 2 g of sodium sulphate — trisodium phosphate salt mixture with 8 ml of n butanol for 30 min

Addition µg	Amine recovered in butanol phase			
	Spermidine		Spermine	
	µg	per cent	µg	per cent
25	22 8	91 2	23 6	94 4
»	23 2	92 8	24 0	96 0
»	22 8	91 2	23 8	95 [illegible]
50	45 6	91 2	47 3	94 6
»	47 0	94 0	47 4	94 8
»	44 6	89 2	47 6	95 2
»	45 4	90 8	47 8	95 6
»	46 9	93 8	47 9	95 8
»	46 2	92 4	48 0	96 0
Mean ± S D		91 8 ± 1 5		95 3 ± 0 6

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amido black (p 39) As shown in Fig 5 1 oamylamine and ornithine migrated near to spermine Owing to the relatively short duration of the run (2 h) used in this experiment lysine also migrated near to spermine 1 3 Diaminopropane putrescine and histamine were distinctly separated from spermidine and spermine

As stated on p 41 ornithine and lysine even in high concentrations did not interfere with the quantitative determination of polyamines with amido black However to achieve a distinct separation between ornithine and spermine as required in isotope experiments in which radioactive ornithine was used 0 07 M citric acid buffer at pH 3 5 was employed for paper electrophoresis (cf p 48) In these conditions ornithine migrated 5 7 cm spermine 7 7 cm and spermidine 9 0 cm in 2 5 hours

Identification of Spermidine and Spermine by Paper and Thin Layer Chromatography

Few solvent systems give an adequate separation of polyamines HERBST KEISTER and WEAVER (1958) tested more than 50 solvent systems and found only two to be useful for the separation of diamines and polyamines viz diethylene glycol monomethyl ether—propionic acid—water (70 15 15) saturated with sodium chloride and ethylene glycol monomethyl ether—propionic acid—water (70 15 15) saturated with sodium chloride The latter was used in the present study and gave a distinct separation of spermidine and spermine The following solvent systems were used in paper chromatography

Solvent 1 ethylene glycol monomethyl ether—propionic acid—water 70 15 15 saturated with sodium chloride
Solvent 2 *n* butanol—acetic acid—water 4 1 5
Solvent 3 buffered phenol pH 1 (buffer 50 ml of 0 2 M KCl and 97 ml of 0 2 N HCl buffer—phenol 1 1
According to FISCHER and BOHN 1957b)

Both one and two dimensional ascending paper chromatography was performed with Whatman No 1 paper Solvents 1 and 3 were used in one dimensional chromatography The average Rf values were as follows solvent 1 spermine 0 20 spermidine 0 31 solvent 3 spermine 0 14 spermidine 0 21 In the two dimensional method

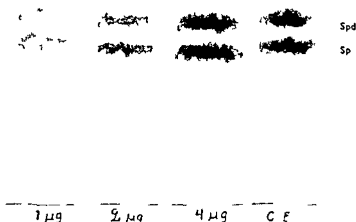


Fig 1 Separation of spermidine and spermine by paper electrophoresis 0 065 M sulphosalicylic acid buffer pH 3 5 Potential gradient 8 V/cm time of electrophoresis 2 5 h Paper Whatman No 1 3 × 39 cm Staining with amido black 1 2 or 4 µg of each polyamine per strip C E extract of 4 day old chick embryo Spd = spermidine Sp = spermine

Paper electrophoretic separation of various amines and basic amino acids The studies in which the paper electrophoretic migration of spermidine and spermine has been compared with that of other amines and basic amino acids have shown that the naturally occurring aliphatic diamines, viz 1,3 diaminopropane, putrescine and cadaverine having a greater mobility, can be distinctly separated from the polyamines with several buffer systems (FISCHER and BOHN 1957b, HERBST KEISTER and WEAVER 1958) Of the various mono amines, butylamine and amylamine had a mobility near to that of the polyamines in a system in which 0 05 M sulphosalicylic acid buffer at pH 4 0 was used (HERBST KEISTER and WEAVER 1958) FISCHER and BOHN (1957b) using pyridine—acetic acid—citric acid buffer systems obtained a good separation of basic amino acids including arginine histidine and lysine from the polyamines Ornithine was not studied in their system Histamine and ethanolamine had a mobility near to that of spermidine

In the present experiments attention was paid to the paper electrophoretic mobility of the basic compounds which would interfere with the quantitative determination of spermidine and spermine with

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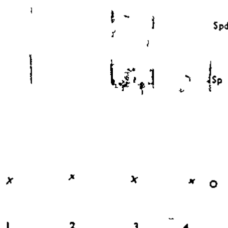


Fig 2 Separation of spermidine and spermine by one dimensional paper chromatography Solvent ethylene glycol monomethyl ether — propionic acid — water, 70 15 15 saturated with sodium chloride Paper Whatman No 1 Ascending technique run for 6 hours Staining with amido black Strip No 1 0 5 μg No 2 1 μg and No 3 2 μg of each polyamine per strip No 4 chromatogram from a 10 day old chick embryo Spd = spermidine Sp = spermine O = origin

solvent 2 was used in the first stage (Rf values spermine 0 04 spermidine 0 07) solvent 1 in the second stage (Rf values spermine 0 07 spermidine 0 18)

For thin layer chromatography the layer of adsorbent (Kieselgel G nach Stahl Merck) was applied to glass plates of 20 × 20 cm with the Desaga applicator (C Desaga Heidelberg) Solvent 1 was used in slightly modified form

Solvent 4 ethylene glycol monomethyl ether—propionic acid—water 77 5 15 7 5

For detection of polyamines the chromatograms were sprayed with 0 2 per cent ninhydrin (in abs ethanol—pyridine—conc hydrochloric acid 94 4 1) or with amido black solution As little as 0 25 μg of each polyamine could be detected with both reagents on paper chromatograms and 0 1 μg on silica gel chromatograms Some typical

paper chromatograms stained with amido black are seen in Fig 2 As shown in Fig 2 (see also Fig 1) spermidine and spermine are the only compounds in chick embryo extracts which formed poorly soluble complexes with amido black

DETERMINATION OF SPERMIDINE AND SPERMINE WITH AMIDO BLACK

Since GRASSMANN and HANNIG (1950) introduced amido black for protein staining many modifications to the method have been suggested especially that the washing procedure should be made more rapid In the present method the washing procedure was in principle the same as was used by GRASSMANN and HANNIG whereas it was necessary to modify the dye solution as well as the staining technique to achieve optimal recoveries In the following experiments the procedure was studied step by step to rule out possible sources of error and to find reasons for the method finally adopted (p 43)

Factors Affecting Staining

Drying of paper strips before staining A slight decrease of 2 to 5 per cent in recoveries was observed when the paper strips were dried in an oven at 105 C for 5 to 60 min before staining compared with the values obtained from papers dried at room temperature For this reason the strips were allowed to stand at room temperature after electrophoresis to lose most of the extra moisture which took about one hour and were then stained

Interference with dye binding on papers stored unstained When sulphosalicylic acid buffer was used for electrophoresis a decrease in the recovery of the dye was sometimes observed In these cases the paper strips had been kept unstained for several days to a few months after electrophoresis This observation led to a more accurate study of this effect From 20 strips run simultaneously 3 or 4 papers at a time were stained at intervals of several days As little as four days later a slight decrease in the recovery of the dye of both the spermidine and spermine fractions could be observed As seen from Fig 3 the decrease became more marked with time

3

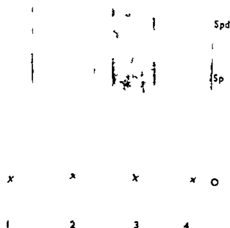


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Table 5 *Dye binding of spermidine and spermine by different staining procedures*

a) spray with saturated amido black in methanol—acetic acid—water (2 1 7) = present method

b) spray with saturated amido black in methanol—acetic acid (9 1)

c) staining for 10 min in dye bath with the same solution as in b)

After staining the papers were dried for 5 min at 105 C followed by washing in methanol—acetic acid (9 1) for 90 min Each absorbance value represents a mean of 4 determinations 4 µg of each polyamine per strip

Amine	Staining technique		
	a	b	c
Spermidine	0.4[illegible]4	0.41[illegible]	0.419
Spermine	0.340	0.[illegible]9[illegible]	0.331

Table 5 However as the polyamines are water soluble staining with aqueous solution in a dye bath was not feasible For this reason spraying of papers with the dye solution was tested and found to be very suitable for the purpose Staining of 20 strips took only a few minutes and consumed little dye solution In addition contamination of the dye with buffer was avoided It was only necessary to spray one side of the paper

After spraying the papers were dried in an oven at 105 C for 5 min No significant differences in recoveries were found when the drying time was varied from 5 to 30 min On the other hand when the strips were washed without previous drying a considerable loss of colour was found in the spermidine fraction

Washing procedure An essential step in the present method is the washing of the excess dye from the paper after staining As is generally known amido black is bound very firmly by cellulose so that to achieve a nearly colourless background a washing time of several hours is required if the usual methanol—acetic acid (9 1) is used For protein staining many modifications of the washing procedure have been introduced *e g* the use of aqueous acetic acid solution (*e g* OOSTERHUIS 1954 LUCAR 1954) In the present method methanol—acetic acid (9 1 vol /vol) was preferred because addition of water causes losses of the spermidine fraction as does the use of methanol alone Prolongation of the washing time naturally gives lower background values but at the same time a possible washing

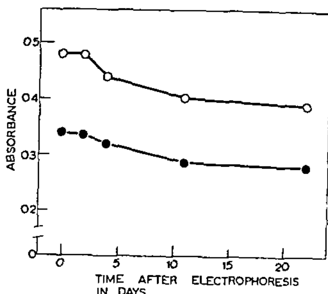


Fig 3 Interference with dye binding on papers stored unstained after electrophoresis Sulphosalicylic acid buffer Each spot represents a mean of 3 or 4 determinations ○ = spermidine ● = spermine 4 µg of each polyamine per strip

Citric acid buffer was also used in a similar experiment but no change in the recovery was observed during 20 days It is probable that oxidized sulphosalicylic acid reacts in some way with the polyamines, so interfering with their dye binding However if the paper strips are stained within one day after electrophoresis, the sulphosalicylic acid buffer can be used without any loss of colour

During electrophoresis, the sulphosalicylic acid buffer became markedly coloured especially in the anode side reservoir When such a coloured buffer was used again a similar »washing off« effect was found to that described above Therefore the sulphosalicylic acid buffer must be changed after every run By contrast the same citric acid buffer can be used several times in succession provided it is always remixed between runs

Staining and Washing Procedures

Staining In protein staining amido black is used as a rule in a saturated methanolic solution with 10 per cent acetic acid In the present experiments, however it was found that staining with an aqueous solution of amido black gave better results as is seen from

Table 5 Dye binding of spermidine and spermine by different staining procedures

a) spray with saturated amido black in methanol—acetic acid—water (2 1 7) — present method

b) spray with saturated amido black in methanol—acetic acid (9 1)

c) staining for 10 min in dye bath with the same solution as in b)

After staining the papers were dried for 5 min at 105 C followed by washing in methanol—acetic acid (9 1) for 90 min Each absorbance value represents a mean of 4 determinations 4 µg of each polyamine per strip

Amine	Staining technique		
	a	b	c
Spermidine	0 474	0 41[illegible]	0 419
Spermine	0 340	0 [illegible]9[illegible]	0 331

Table 5 However as the polyamines are water soluble staining with aqueous solution in a dye bath was not feasible For this reason spraying of papers with the dye solution was tested and found to be very suitable for the purpose Staining of 20 strips took only a few minutes and consumed little dye solution In addition contamination of the dye with buffer was avoided It was only necessary to spray one side of the paper

After spraying the papers were dried in an oven at 105 C for 5 min No significant differences in recoveries were found when the drying time was varied from 5 to 30 min On the other hand when the strips were washed *without previous drying a considerable loss of* colour was found in the spermidine fraction

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off of the polyamine fractions must be taken into account. In Table 6 the effect of the washing time is shown. This indicates that the amido black spermine complex is practically insoluble in methanol—acetic acid and very firmly attached to the paper. By contrast, small losses can be observed in the spermidine fraction, for in this case prolongation

Table 6. Effect of washing time on the dye binding of spermidine and spermine on paper. 4 μg of each polyamine per strip. The absorbance values represent means of 4 determinations

Washing time min	Absorbance		
	Spermidine — blank	Spermine — blank	Blank
60	0.479	0.349	0.065
75	0.481	0.348	0.058
90	0.471	0.349	0.048
120	0.461	0.350	0.045
150	0.449	0.353	0.040
180	0.444	0.356	0.034

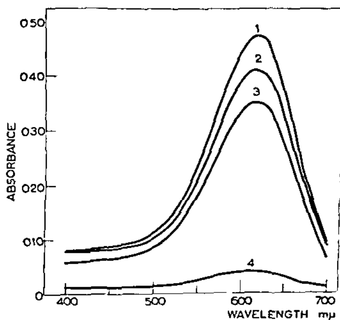


Fig. 4. Absorption spectra of amido black in 0.1 N NaOH measured in 1 cm cells against water. No. 2: 10 μM authentic amido black. No. 1, 3, 4: absorption spectra of the dye eluted from a paper electrophoretic strip with 5 ml of 0.1 N NaOH. No. 1: 4 μg of spermidine (— blank). No. 3: 4 μg of spermine (— blank). No. 4: blank

of the washing time from 60 to 180 min caused a fall of 7 per cent in the recovery A washing time of 90 min was chosen since thereafter the background colour decreased relatively little and on the other hand the background was quite evenly decolourized

In this connexion it can be mentioned that spermidine and spermine give different colours with amido black on paper The amido black spermidine complex is greyish whereas with spermine amido black gives a bluish colour However the absorption spectra of the amido black polyamine complexes eluted with sodium hydroxide solution from paper are identical with that of authentic amido black showing an absorption maximum at 615 mμ (Fig 4) This indicates that the complexes are dissociated in alkaline solution

Dye binding Capacity of Spermidine and Spermine

The molar ratio (amido black/polyamine) in the amido black polyamine complex was determined as follows

To determine the molar extinction coefficient of amido black the dye was carefully dried to constant weight and 4 samples of 572 mg (1 mmole) each were dissolved in 1 l of 0 1 N sodium hydroxide and further diluted to 1 100 with the same solution This diluted solution (10^{-5}M) gave a mean absorbance of 0 418 in 1 cm cells at 615 mμ against water The molar extinction coefficient calculated from this value was 41 800/mole/l at 615 mμ The corresponding extinction values of amido black bound to one mole of spermidine or spermine on paper were 85 200 and 85 900 respectively (Table 7) This indicates that two moles of amido black are bound per mole of polyamine

Table Dye binding capacity of spermidine and spermine on paper Calculated from extinction of amido black bound per mole of polyamine on paper dissolved in 1 l of 0 1 N sodium hydroxide Molar extinction coefficient of amido black 41 800 at 615 mμ

	Extinction of amido black bound per mole polyamine	Mole of amido black bound per mole of polyamine
Spermidine	85 200	2 04
Spermine	85 900	2 05

Since the amido black spermine complex appeared to be insoluble in water, an experiment was made in which spermine was precipitated with amido black in aqueous solution. 0.1 mmole of spermine was precipitated with 0.4 mmole of amido black in 10 ml of distilled water, and 20 ml of methanol—acetic acid (9 : 1) was added at + 3°C. After two hours the precipitate was separated by centrifugation and washed ten times with 20 ml of methanol—acetic acid mixture, the supernatant was then practically colourless. The precipitate was collected, dried at 105°C and weighed. The yield was 140.0 mg. If it is assumed that the spermine was precipitated quantitatively, the amount of amido black in the amido black spermine complex would be

140.0 mg — *20.2* mg (0.1 mmole of spermine) = 119.8 mg. 119.8 mg is equal to 0.209 mmole of amido black. Thus this result is in agreement with the ratio calculated above from the molar extinction coefficient.

Because the amido black spermidine complex is slightly soluble in water, spermidine was not precipitated quantitatively in the above mentioned conditions.

Stability of Amido Black in Alkaline Solution

PEZOLD and PEISER (1953), using amido black for protein staining observed a marked and rapid decrease in the colour of amido black in 0.1 N sodium hydroxide solution after elution of the dye from the paper, especially if the concentration of stained protein was high. A similar instability of the colour was also found by MONCKE (1956) in experiments in which the protein was fixed by heating, the colour remained constant if methanol—ether was used for fixation. The decrease in colour was greatest at the absorption maximum while at 500 to 520 mμ it remained constant (EBERHARD 1956).

In contrast, in the course of the present work amido black was not found to be unstable in alkaline solution after elution of the dye from the paper. During the first 3 to 4 hours there was no significant change in the colour measured at the absorption maximum (615 mμ). Similarly, pure amido black dissolved in 0.1 N sodium hydroxide showed no instability during 6 hours. In this connexion it may be emphasized that according to DITTMER (1961, p. 88) the colour remains unchanged for two hours after elution from the paper.

According to OSBORN (1960) the important combining sites of protein with acidic dyes are the basic groups of histidine lysine and arginine To test the degree to which various basic substances other than spermidine and spermine e g other amines and basic amino acids would be able to form poorly soluble complexes with amido black a rapid screening on paper was carried out with relatively strong solutions 20 to 40 μg of each compound (expressed as free base or as salt) was applied in acid solution onto paper dried and stained with amido black The commercial preparations tested included

Glutamine (L Light & Co Ltd Colnbrook Bucks) asparagine (Merck) ornithine (Fluka) arginine (B D H) lysine (Merck) histidine methylamine diethylamine (all from Hoffmann — La Roche) propylamine (Fluka) isoamylamine ethanolamine (both from Hoffmann — La Roche) tryptamine (Sigma Chemical Co St Louis) serotonine (Hoffmann — La Roche) noradrenalin (Light) histamine (B D H) 1 3 diaminopropane (Th Schuchardt GMBH & Co Munchen) putrescine cadaverine adenine and uracil (all from Hoffmann — La Roche)

Eight of these including ornithine arginine lysine histamine propylamine isoamylamine 1 3-diaminopropane and putrescine gave a more or less sparingly soluble precipitate with amido black These compounds were then subjected in amounts of 4 μg of each expressed as free base to paper electrophoresis along with 2 μg of spermidine and spermine One of the duplicate sheets was stained with amido black and the other with ninhydrin (Fig 5) All the fractions other than the polyamines were gradually washed off After a washing time of 90 min a weak spot of amido black was left in the histamine area whilst only negligible traces of amido black could be found in the 1 3 diaminopropane putrescine ornithine arginine and lysine areas The propylamine and isoamylamine precipitates were washed off completely Of the various diamines tested 1 3 diaminopropane formed the least soluble complex with amido black whereas the cadaverine amido black precipitate was readily washed off from the paper

These results indicate that the specificity of the amido black method for spermidine and spermine is not absolute even though of high degree Therefore butanol extraction and paper electrophoresis

Since the amido black spermine complex appeared to be insoluble in water an experiment was made in which spermine was precipitated with amido black in aqueous solution 0 1 mmole of spermine was precipitated with 0 4 mmole of amido black in 10 ml of distilled water, and 20 ml of methanol—acetic acid (9 1) was added at + 3°C After two hours the precipitate was separated by centrifugation and washed ten times with 20 ml of methanol—acetic acid mixture the supernatant was then practically colourless The precipitate was collected dried at 105°C and weighed The yield was 140 0 mg If it is assumed that the spermine was precipitated quantitatively the amount of amido black in the amido black spermine complex would be

140 0 mg — 20 2 mg (0 1 mmole of spermine) = 119 8 mg 119 8 mg is equal to 0 209 mmole of amido black Thus this result is in agreement with the ratio calculated above from the molar extinction coefficient

Because the amido black spermidine complex is slightly soluble in water spermidine was not precipitated quantitatively in the above mentioned conditions

Stability of Amido Black in Alkaline Solution

PEZOLD and PEISER (1953) using amido black for protein staining observed a marked and rapid decrease in the colour of amido black in 0 1 N sodium hydroxide solution after elution of the dye from the paper especially if the concentration of stained protein was high A similar instability of the colour was also found by MONCKE (1956) in experiments in which the protein was fixed by heating the colour remained constant if methanol—ether was used for fixation The decrease in colour was greatest at the absorption maximum while at 500 to 520 mμ it remained constant (EBERHARD 1956)

In contrast in the course of the present work amido black was not found to be unstable in alkaline solution after elution of the dye from the paper During the first 3 to 4 hours there was no significant change in the colour measured at the absorption maximum (615 mμ) Similarly, pure amido black dissolved in 0 1 N sodium hydroxide showed no instability during 6 hours In this connexion it may be emphasized that according to DITTMER (1961 p 68) the colour remains unchanged for two hours after elution from the paper

electrophoresis (Fig 5) However the bulk of these basic amino acids remains in the aqueous phase in butanol extraction and does not interfere with the determination of spermidine and spermine This was demonstrated by adding ornithine and lysine to the protein free tissue extract in amounts corresponding to a tissue concentration of 0 5 per cent (4 mg to 8 ml) prior to butanol extraction and analysing as described on p 43 This addition had no effect on the recovery of spermidine and spermine

In conclusion it may be stated that the present method which involves butanol extraction subsequent paper electrophoresis and in addition quantitative determination with amido black is highly specific for the determination of spermidine and spermine

Precision and Sensitivity

Assuming that the main sources of error discussed in the preceding sections are eliminated the amido black method for the determination of spermidine and spermine is quite reproducible Thus twenty one standards run in a week 4 μg of each polyamine per strip gave a mean absorbance of 0 470 $\pm$ 0 006 (standard deviation) for spermidine and 0 343 $\pm$ 0 005 for spermine At the level of 2 μg of each polyamine a standard deviation of $\pm$ 2 1% for spermidine and $\pm$ 1 9% for spermine was found at the same time Even at the level of 0 5 to 1 μg of polyamine the duplicates did not as a rule deviate by more than 2 to 3 per cent from the mean Hence with this method quantities of 0 5 to 1 μg of polyamine still permitted an accurate determination provided that in these cases the concentration of the standard was near to that of the unknown sample Qualitatively as little as 0 2 μg of polyamine could be detected

To check the precision of the present method as applied to the determination of polyamines from tissue ten simultaneous analyses were made from the same tissue homogenate The result is shown in Table 8 The standard deviation was $\pm$ 1 9% for spermidine and $\pm$ 2 1% for spermine

Calibration curves for spermidine and spermine are given in Fig 6 The amount of polyamine is expressed as mμmoles since in the following part of this work calculation of the polyamine content in tissue as molar concentration was preferred Absorption was linear up to at least 50 mμmoles of polyamine The calibration curve for

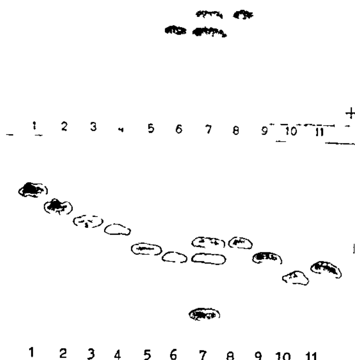


Fig 5 Separation of certain amines and amino acids by paper electrophoresis 0 065 M sulphosalicylic acid buffer pH 3 5 2 h at 300 V paper Whatman No 1 26 × 39 cm Upper sheet stained with amido black lower sheet with ninhydrin 4 µg (2 µg of spermidine and spermine) of the following compounds expressed as free base were applied (electrophoretic migration in cm in parentheses)

1	*1 3 diaminopropane*	*(10 7)*	*6*	*spermine*	*(6 3)*
2	*putrescine*	*(9 5)*	*8*	*spermidine*	*(7 4)*
3	*propylamine*	*(8 7)*	*9*	*ornithine*	*(6 3)*
4	*histamine*	*(8 2)*	*10*	*arginine*	*(5 0)*
5	*isoamylamine*	*(6 8)*	*11*	*lysine*	*(5 8)*

Sample No 7 extract of chick embryo liver corresponding to 20 mg of fresh tissue In addition to spermine (6 3 cm) and spermidine (7 3 cm) a weak ninhydrin positive fraction is seen in the »putrescine area» (9 4 cm)

are required to achieve complete specificity As previously stated (p 31) *1 3 diaminopropane putrescine and histamine were distinctly* separated from the polyamines whereas of the basic amino acids, ornithine and lysine had a mobility near to that of spermine on paper

spermidine passed slightly below the origin. This was due to a small loss of about one mμmole in the spermidine fraction during the washing procedure. With a standardized procedure this loss was found to remain constant.

THE PRESENT METHOD

A summary of the detailed procedure for the determination of spermidine and spermine in tissue based on the experiments described in the preceding part is given below.

Reagents. *Salt mixture for butanol extraction.* 62.5 g of anhydrous sodium sulphate and 9 g of trisodium phosphate are mixed by grinding in a mortar and stored in a vacuum desiccator.

Saturated amido black in methanol—acetic acid—water (2 : 1 : 7). Saturated solution is prepared by allowing a suspension to stand overnight and then filtering. This solution is stable for several months at room temperature.

Methanol—glacial acetic acid (9 : 1 vol./vol.) mixture for washing. Technical methanol redistilled at 64—65°C was found useful for this purpose.

Buffers. 0.065 M *sulphosalicylic acid buffer* pH 3.5: 16.5 g of sulphosalicylic acid is dissolved in distilled water, 30.6 ml of 4 N NaOH is added, and diluted with distilled water ad 1000 ml. 0.1 M *citric acid buffer* pH 3.5: 21.0 g of citric acid is dissolved in distilled water, 20 ml of 4 N NaOH is added, and diluted with distilled water ad 1000 ml.

Reference standards. Polyamine solutions containing 1, 2 or 4 μg of spermidine and spermine in 20 μl of 0.1 N HCl.

Procedure

One part of tissue is homogenized with the Ultra Turrax homogenizer in 4 parts (wt./vol.) of 0.1 N hydrochloric acid for 40 sec. 5 ml of homogenate is deproteinized by addition of an equal volume of 10 per cent trichloroacetic acid, and the mixture shaken several times and filtered after about one hour. 8 ml of the filtrate is transferred to a glass stoppered test tube and extracted three times with an equal volume of ether to remove most of the trichloroacetic acid. With the aid of a metal rack 30 to 40 samples can be treated simultaneously.

Table 8 Spermidine and spermine content determined in ten simultaneous analyses from the same tissue homogenate of 15 day old chick embryos Contents of polyamines are expressed as µg/g wet weight

No	Concentration µg/g wet wt	
	Spermidine	Spermine
1	77 9	85 1
2	79 3	86 5
3	81 5	84 6
4	81 0	85 1
5	79 0	83 8
6	79 0	88 3
7	79 0	88 9
8	77 4	84 6
9	77 1	88 6
10	77 1	86 2
Mean ± S D	*78 8 ± 1 5*	*86 2 ± 1 9*

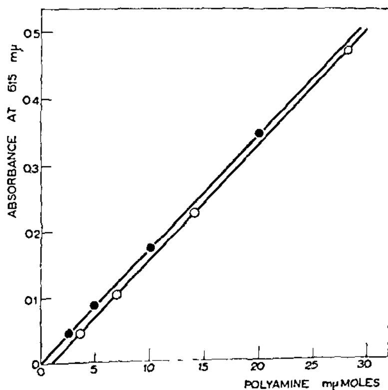


Fig 6 Calibration curves for spermidine and spermine Absorbance at 615 mµ Abscissa mµmoles of polyamine in 20 µl sample applied to paper electrophoresis ○ = *spermidine* ● = *spermine*

The present method for the quantitative determination of spermidine and spermine from tissues consists principally of three steps viz 1) partial purification of the amine fraction from protein free tissue extracts by butanol extraction 2) paper electrophoretic separation of amines and 3) quantitative determination of spermidine and spermine with amido black Every step has been commented on to some extent in the preceding sections and therefore only some aspects are discussed here

Butanol extraction serves as a rapid procedure for preliminary purification of the amine fraction and gives relatively good recoveries The steam distillation method used by some authors is more time consuming requiring the collection of a large amount of distillate which then has to be evaporated before electrophoresis (HERBST WEAVER and KEISTER 1958) The percentage recoveries of polyamines about 92 per cent for spermidine and 95 per cent for spermine in the butanol extraction were constant which made the use of standards in this step unnecessary One disadvantage of butanol extraction was that when the polyamine content of the sample was very low and considerable concentration was therefore needed before electrophoresis the small amount of salt mixture which always passed into the butanol phase interfered with accurate analysis

Paper electrophoretic separation of polyamines is a reliable simple and fairly rapid method allowing the performance of several runs daily For accurate work careful application of the sample is necessary Sulphosalicylic acid and citric acid buffers are useful for separation of polyamines HERBST KEISTER and WEAVER (1958) used 0.05 M sulphosalicylic acid buffer at pH 4.0 and 0.03 M citric acid buffer at pH 6.5 for qualitative detection of various aliphatic amines It was found in the present study that somewhat higher buffer concentrations are required for quantitative analyses Distinct separation and compact bands were obtained with 0.065 M sulphosalicylic acid buffer at pH 3.5

The present amido black method for quantitative determination of spermidine and spermine is accurate sensitive and fairly specific for polyamines In the handling of the paper strips the great caution necessary with ninhydrin methods is not required Further ammonia does not interfere with this method As little as 5 mμmoles of polyamine

Ether is removed with a thin pipette connected to a vacuum and finally careful warming The sample is then treated with 2 g (measured with a calibrated metal cup) of the salt mixture, the salt being dis ol ved by slight warming To make the sample strongly alkaline 0 4 ml of 4 N NaOH is finally added The alkaline salt mixture is then ex tracted with 8 ml of *n* butanol with vigorous shaking for 30 min in a mechanical shaker After centrifugation of the tubes at low speed for 15 min, the butanol layer is carefully sucked into a pipette with a rubber bulb, transferred to a small porcelain dish, acidified with a few drops of conc hydrochloric acid and then evaporated to dryness *in a vacuum oven at 70 to 80°C* The residue is dissolved with 400 μl of 0 1 N HCl and stored in a small test tube until paper electrophoresis is performed

Paper electrophoresis The paper strips 3 × 39 cm of *Whatman* No 1 are dipped in buffer solution and then blotted to remove the extra moisture The buffer remaining in the paper is about 1 2 to 1 4 times the original weight of the paper The strips are then placed on the rack and with a micropipette 20 μl of the sample is applied on the starting line, 2 cm to the anode side of the centre of the strip the margins of the strip being avoided A constant potential of 300 V corresponding to a potential gradient of about 8 V/cm is applied, giving a sufficient resolution within 2 5 to 3 hours After the electro phoresis is finished the ends of the papers are blotted and the strips allowed to dry at room temperature until stained

Staining and photometric evaluation The paper strips are sprayed with amido black solution and then dried at 105°C for 5 min The excess dye is then removed by washing in a methanol—acetic acid bath for exactly 90 min the washing solution being changed at intervals of 10 20 and 30 min (the last two 30 min each) After a final drying of the strips for 5 min at 105°C the polyamine fractions and the blank each of equal size are cut off and the dye is eluted with 5 ml of 0 1 N NaOH for 20 to 30 min in a test tube shaken twice during the elution The colour is measured within one hour after elution in a Beckman B spectrophotometer at 615 mμ using cells with a 1 cm light path

The polyamine content of the unknown sample is read from a calibration curve obtained from the reference standards run simulta neously with unknown samples in electrophoresis Corrections of 8 per cent for spermidine and of 5 per cent for spermine are applied on account of losses incurred during the butanol extraction

Incubation of Eggs

Fertilized eggs of White Leghorn chickens weighing 55±2 g were obtained from a commercial source (Saarioinen Oy Sahalahti) and incubated at 38±0 5 C in a relative humidity of 65±5 per cent with forced air circulation The eggs were placed on racks with the air space upwards The racks each containing 30 eggs were kept tilted at an angle of about 30 degrees to the horizontal and turned once daily

Injection Technique

Before injection the eggs were tran illuminated to make sure of the viability of the embryos The blunt end of the egg was then swabbed with 70 per cent ethyl alcohol All injections were made into the air space (onto the chorio allantoic m mbrane) through a small hole drilled in the shell straight above the air space in a volume of 100 to 200 μl After the injection the hole was sealed with adhesive tape The injected eggs were kept in an upright po ition and not turned thereafter

Preparation of Homogenates

Homogenates of whole embryos were prepared in the following manner embryos were removed with forceps freed from the adherent membranes and yolk sac and then dried gently with blotting paper Small embryos were removed from the eggs with a spoon and prepared on a cooled sheet of steel The embryos were then weighed and homogenized with the Ultra Turrax homogenizer for 40 sec in 0 1 N HCl one part of fresh tissue to 4 parts (wt /vol) of hydrochloric acid Embryos 10 days old or younger were homogenized in a Potter Elvehjem type homogenizer for 2 min This technique was also used in the isotope experiments The polyamine contents obtained from 10-day embryos with these two different homogenization techniques were the same

A suitable number (2 to 90) of small embryos were pooled for the determination of both the polyamine content and dry weight of the same sample Homogenates from individual organs were prepared in a similar manner 1 to 10 organs being pooled and homogenized with the Ultra Turrax homogenizer

Analytical Methods

Determination of relative dry weight 5 ml of homogenate was pipetted into a small beaker weighed and dried in an oven at 105 C usually for two days to constant weight For calculation of the relative dry weight D (mg/g wet weight) the following equation was used (weights as mg)

D a b × d c where
a = weight of homogenate containing 1 g of fresh tissue
b = wet weight of the sample
d = dry weight of the sample
c = weight of hydrochloric acid per a mg of homogenate

could be determined accurately with amido black. Thus the present method is at least as sensitive as the ninhydrin methods (FISCHER and BOHN 1957b, WEAVER and HERBST 1958), if not more so. The dinitrofluorobenzene method reported by DUBIN (1960) also allowed the determination of quantities as small as 10 mμequivalents of amino groups but this method as such is not applicable to paper.

The principle of the present quantitative method is precipitation of the polyamines with amido black on paper. As previously stated two moles of the dye are bound to one mole of either polyamine, which seems to indicate that the amount of dye bound is determined by the number of primary amino groups in these amines. This does not, however, exclude the possibility that the secondary amino groups — one in spermidine and two in spermine — also participate in the complex formation. In fact, the differences in the colours and solubilities of the amido black-polyamine complexes, which are described in the previous sections, appear to support the latter assumption. The amido black-polyamine precipitate is amorphous and strikingly firmly attached to the paper, especially in the spermine fraction. Although the complex is very poorly soluble in a methanol—acetic acid mixture, it readily dissolves in alkaline solution, in which the complex is dissociated. It is therefore possible to use amido black precipitation for purification of the polyamines as described in connexion with the isotope experiments (p. 48).

METABOLISM OF SPERMIDINE AND SPERMINE IN THE DEVELOPING CHICK EMBRYO

MATERIAL AND METHODS

The chick embryo was chosen as the experimental object for the present study on the basis of preliminary experiments. For it was found that neither spermidine nor spermine could be detected in unincubated eggs or in their hydrolysates, whereas both these amines were present in embryos, indicating synthesis during embryonic development. The chick embryo was therefore considered to be suitable for studying the biosynthesis of polyamines.

Incubation of Eggs

Fertilized eggs of White Leghorn chickens weighing 55±2 g were obtained from a commercial source (Saarioinen Oy Sahalahti) and incubated at 38±0.5 C in a relative humidity of 65±5 per cent with forced air circulation The eggs were placed on racks with the air space upwards The racks each containing 30 eggs were kept tilted at an angle of about 30 degrees to the horizontal and turned once daily

Injection Technique

Before injection the eggs were transilluminated to make sure of the viability of the embryos The blunt end of the egg was then swabbed with 70 per cent ethyl alcohol All injections were made into the air space (onto the chorio allantoic membrane) through a small hole drilled in the shell straight above the air space in a volume of 100 to 200 μl After the injection the hole was sealed with adhesive tape The injected eggs were kept in an upright position and not turned thereafter

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Determination of relative dry weight 5 ml of homogenate was pipetted into a small beaker weighed and dried in an oven at 105 C usually for two days to constant weight For calculation of the relative dry weight D (mg/g wet weight) the following equation was used (weights as mg)

$D = a/b \times d - c$ where
a = weight of homogenate containing 1 g of fresh tissue
b = wet weight of the sample
d = dry weight of the sample
c = weight of hydrochloric acid per a mg of homogenate

Without noteworthy error it was used in the following form

$$D = 5\,000/b \times d - 14.6$$

Determination of spermidine and spermine Details of the method are given on p 43

Isotope Experiments

Radioactive material The following ^{14}C labelled compounds were used

— putrescine 1 4 ^{14}C dihydrochloride specific activity 4 17 mC/mmole (New England Corporation Boston Massachusetts)
— DL ornithine 2 ^{14}C hydrochloride specific activity 1 0 mC/mmole (California Corporation for Biochemical Research Los Angeles California)
— DL methionine 2 ^{14}C specific activity 1 27 mC/mmole (Volk Radiochemical Company Skokie Illinois)

The labelled compounds were dissolved in 0 9 per cent sodium chloride and injected in a volume of 100 to 200 μl

Liquid scintillator »Scintipak 2« (Nash & Thompson Ltd Tolworth) was used as phosphor for liquid scintillation counting It was dissolved in an appropriate volume of dioxane (für Chromatographie Merck) As an alternative solvent a mixture containing dioxane xylene (für Chromatographie Merck) abs ethyl alcohol and conc hydrochloric acid (*p a* Merck) according to BOUSQUET and CHRISTIAN (1960) was tested These solvents gave very similar results

Preparation of samples and radioactivity measurements The isolation of spermidine and spermine from tissues was largely the same as the isolation of unlabelled polyamines (p 43) However to achieve a strict separation of spermidine and spermine 0 06 M (instead of 0 065 M) sulphosalicylic acid or 0 07 M (instead of 0 1 M) citric acid buffer of pH 3 5 was used in isotope experiments The latter buffer was used especially in experiments with ^{14}C labelled ornithine to separate ornithine distinctly from spermine

After paper electrophoretic separation the paper strips to be counted were sprayed with ninhydrin solution and kept in an oven at 105 C until the coloured fractions just appeared The fractions usually 12×30 mm were cut off and counted directly from the papers in a liquid scintillation counter by placing these freely on the bottom of the counting vials parallel to the face of the photomultiplier tube This technique was simple and gave reproducible results (cf BOUSQUET and CHRISTIAN 1960 BLAIR and SEGAL 1962) A linear relation between the counts applied and yielded was obtained up to at least 10 000 cpm For calculation of specific activity spermidine and spermine were determined quantitatively from duplicate strips with amido black

Counting was carried out in an Ekco type 664 A liquid scintillation counter (Ekco Electronics Ltd) with 10 ml of liquid scintillator in the counting vials (Ekco type N671A coated containers) The samples were counted to give a counting error of less than 2 per cent

The radiopurity of the spermidine and spermine fractions was tested as follows The polyamines were separated by paper electrophoresis and stained with amido black The coloured fractions were cut off and eluted with 0 1 N

sodium hydroxide and the polyamines extracted into n butanol while amido black remained in the aqueous phase After the second electrophoresis the specific activity of spermidine and spermine was essentially the same as after the first separation

Autoradiographic technique The electrophoretic paper strips stained with amido black were exposed to the films (Kodak »Industrex» D Kodak Ltd. London) and placed in a lightproof box The exposure time varied from 10 to 30 days

The films were developed in »Kodak» D 19b Developer for 5 min rinsed in water and fixed in »Kodak Rapid Acid Fixer The films were then washed for 20 min in running water and dried

Statistical Procedures

The mean values presented are arithmetic means ± S D (standard deviation)

The experimental groups were compared by means of the *t* test The *t* values were calculated according to BANCROFT (1957 p 1 6) it being assumed that the variations of the groups to be compared are basically the same

OCCURRENCE OF SPERMIDINE AND SPERMINE IN THE CHICK EMBRYO

In preliminary experiments unincubated hen s eggs were analysed for their polyamine contents the yolk and the albumen being analysed separately As mentioned before neither spermidine nor spermine could be detected in the eggs before or after acid hydrolysis This indicates that the polyamine contents of the yolk and the albumen are less than 1 mμmole per g of wet matter It is therefore evident that the polyamines detected in relatively high concentrations in the chick embryo cannot be derived in appreciable amounts from the egg

In the following experiments the polyamine contents of the whole chick embryos were determined at different stages of development Analyses of whole embryos were made at intervals of two days from the third day of incubation The polyamine contents were calculated per unit of embryo wet weight and dry weight The results for several incubation batches are summarized in Table 9 and discussed in greater detail in the following section In addition the polyamine contents were determined separately from some tissues of the 18 day old embryos In the following the expression embryo age has been used in the same sense as incubation time

Table 9 Weight relative dry weight and polyamine contents of the chick embryo at different stages of development Relative dry weight expressed as mg per g of wet weight the polyamine contents as mμmoles per g of wet weight and per 100 mg of dry weight Mean values ± S D (standard deviation) Spd = spermidine Sp = spermine

Age in days	Series	Number of embryos	Wt of embryos g	Relat dry wt mg/g w wt	Mean content of polyamine: mμM/g wet wt		Mean content of polyamine: mμM/100 mg dry wt	
					Spd	Sp	Spd	Sp
2 2/3	20	1* (90)	0 004	60	611	488	1020	813
4	16	1* (24)	0 059	57	488	397	856	69[illegible]
6	12	3* (4)	0 312	61	356	321	587	5[illegible]6
"	17	2* (3)	0 367	60	340	331	567	552
Total		5*	0 335 ± 0 043	61 ± 1	350 ± 9	325 ± 15	579 ± 13	536 ± 1[illegible]
8	12	3* (2)	1 026	62	324	298	525	483
"	17	2* (2)	1 170	62	342	305	556	496
Total		5*	1 084 ± 0 101	62 ± 1	331 ± 17	301 ± 19	538 ± 26	488 ± 30
10	9	5	1 74 ± 0 08		306 ± 12	292 ± 12		
"	18	6	2 30 ± 0 18	66 ± 4	308 ± 22	292 ± 21	466 ± 1[illegible]	442 ± 15
Total		11	2 04 ± 0 32	66 ± 4	307 ± 18	292 ± 16	466 ± 15	44[illegible] ± 15
12	17	6	4 97 ± 0 09	81 ± 3	377 ± 1[illegible]	376 ± 15	46[illegible] ± 1[illegible]	466 ± 11
14	12[1]	6	8 37 ± 0 42	100 ± 7	504 ± 56	394 ± 40	50[illegible] ± 34	393 ± 19
"	17	3	9 18 ± 0 35	111 ± 7	484 ± 19	453 ± 34	43[illegible] ± 21	408 ± 8
"	18[2]	7	[illegible]42 ± 0 46	111 ± 3	[illegible]30 ± 15	438 ± 16	4[illegible]6 ± 18	393 ± 19
Total		16	8 98 ± 0 64	10[illegible] ± 8	512 ± 30	424 ± 38	4[illegible]8 ± 34	39[illegible] ± 18
15	11	5	11 03 ± 1 57	141 ± 8	584 ± 19	459 ± 19	[illegible] ± 30	3[illegible]6 ± 1[illegible]
"	12	[illegible]	11 [illegible]1 ± 0 69	137 ± 5	601 ± [illegible]2	423 ± 30	439 ± 21	309 ± 24
"	18[3]	6	11 [illegible]4 ± 1 36	129 ± [illegible]	[illegible] ± [illegible]4	439 ± 41	443 ± 2[illegible]	341 ± 38
Total		18	11 29 ± 1 15	136 ± 8	586 ± 24	438 ± 33	434 ± 2[illegible]	3[illegible]4 ± 29
16	10	6	14 61 ± 1 3[illegible]	1[illegible]6 ± 11	613 ± [illegible]	4[illegible]9 ± 45	349 ± 1[illegible]	[illegible] ± 16
"	17	3	14 69 ± 1 00	170 ± [illegible]	[illegible] ± 14	404 ± [illegible]	340 [illegible]	238 ± 5
"	23a	7	14 82 ± 0 72	18[illegible] ± [illegible]	[illegible]70 ± 2	421 ± 11	313 [illegible] 14	234 ± 7
"	23b	[illegible]	14 [illegible]1 ± 0 [illegible]2	1[illegible]4 [illegible] 9	[illegible]4[illegible] ± 29	402 [illegible] 1[illegible]	314 [illegible] 1[illegible]	[illegible]31 ± 12
Total		23	14 [illegible]1 ± 0 88	1[illegible] ± 8	[illegible] ± 40	4[illegible]6 ± 36	3[illegible]6 ± [illegible]1	4[illegible] ± 18
18	17	6	19 7[illegible] ± 1 94	18[illegible] ± 9	373 ± [illegible]	[illegible]4 ± 16	[illegible]00 ± [illegible]	[illegible]

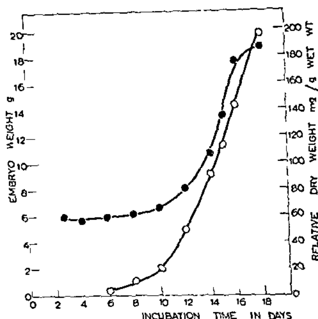


Fig 7 Embryo weight and relative dry weight in relation to embryo age Relative dry weight expressed as mg per g of wet weight ○ = *wet weight* ● = *relative dry weight*

Wet weight and relative dry weight of embryos The wet weights (g) and relative dry weights (mg/g of wet weight) of embryos from different incubation batches are presented in Table 9 and the mean values plotted against the embryo age in Fig 7 The dispersion in values obtained from different batches is fairly small and can be explained by minor variations in the incubation conditions incubation time and analysing technique

Contents of Spermidine and Spermine in the Whole Chick Embryo at Different Stages of Development

The mean spermidine and spermine contents of the whole chick embryos from different incubation batches are presented in Table 9

— = pooled sample number of embryos per pooled sample in parentheses
a = homogenized in 0 1 N HCl
b = homogenized in 0 01 N HCl
1 2 3 = treated 1 2 or 3 times with 900 µl of 0 9 per cent sodium chloride

Table 9 Weight relative dry weight and polyamine contents of the chick embryo at different stages of development Relative dry weight expressed as mg per g of wet weight the polyamine contents as mμmoles per g of wet weight and per 100 mg of dry weight Mean values ± S D (standard deviation) Spd = spermidine Sp = spermine

Age in days	Series	Number of embryos	Wt of embryos g	Relat dry wt mg/g w wt	Mean content of polyamine			
					mμM/g wet wt		mμM/100 mg dry wt	
					Spd	Sp	Spd	Sp
2 2/3	20	1* (90)	0 004	60	611	488	1020	813
4	16	1* (24)	0 059	57	488	397	856	69
6	12	3* (4)	0 312	61	356	321	587	5[illegible]6
”	17	2* (3)	0 367	60	340	331	567	552
Total		5*	0 335 ± 0 043	61 ± 1	350 ± 9	325 ± 15	579 ± 13	536 ± 18
8	12	3* (2)	1 026	62	324	298	525	483
”	17	2* (2)	1 170	62	342	30[illegible]	556	496
Total		5*	1 084 ± 0 101	62 ± 1	331 ± 17	301 ± 19	538 ± 26	488 ± 30
10	9	5	1 74 ± 0 08		306 ± 12	292 ± 12		
”	18	6	2 30 ± 0 18	66 ± 4	308 ± 2[illegible]	292 ± 21	466 ± 1[illegible]	442 ± 15
Total		11	2 04 ± 0 32	66 ± 4	307 ± 18	292 ± 16	466 ± 15	442 ± 15
12	17	6	4 97 ± 0 09	81 ± 3	377 ± 15	3[illegible]6 ± 15	468 ± 12	466 ± 11
14	12[1]	6	8 37 ± 0 42	100 ± 7	504 ± 56	394 ± 40	502 ± 34	3[illegible]3 ± 19
”	17	3	9 18 ± 0 35	111 ± 7	484 ± 19	453 ± 34	437 ± 21	408 ± 8
”	18	7	9 42 ± 0 46	111 ± 3	[illegible]30 ± 1[illegible]	438 ± 16	4[illegible]6 ± 18	393 ± 19
Total		16	8 98 ± 0 64	107 ± 8	512 ± 39	424 ± 38	478 ± 34	396 ± 18
15	11	5	11 03 ± 1 57	141 ± 8	584 ± 19	459 ± 19	41[illegible] ± 30	3[illegible]6 ± 1[illegible]
”	12	7	11 51 ± 0 69	137 ± 5	601 ± 22	423 ± 30	439 ± 21	309 ± 24
”	18[3]	6	11 24 ± 1 36	129 ± [illegible]	[illegible]72 ± 24	439 ± 41	443 ± 26	341 ± 38
Total		18	11 29 ± 1 15	136 ± 8	586 ± 24	438 ± 33	434 ± [illegible]	324 ± [illegible]
16	10	6	14 61 ± 1 32	1[illegible]6 ± 11	613 ± [illegible]	469 ± 4[illegible]	349 ± 1[illegible]	[illegible]67 ± 16
”	17	3	14 69 ± 1 00	170 ± [illegible]	[illegible]79 ± 14	404 ± [illegible]	340 ± [illegible]	[illegible]38 ± [illegible]
”	23a	7	14 82 ± 0 [illegible]2	182 ± [illegible]	[illegible]0 ± [illegible]2	421 ± 11	31[illegible] ± 14	234 ± [illegible]
”	23b	7	14 [illegible]1 ± 0 72	1[illegible]4 ± 9	548 ± [illegible]9	40[illegible] ± 1[illegible]	314 ± 1[illegible]	[illegible]31 ± 12
Total		23	14 71 ± 0 88	17[illegible] ± 8	[illegible] ± 40	4[illegible]6 ± 36	326 ± [illegible]1	[illegible]42 ± 18
18	17	6	19 [illegible]5 ± 1 94	18[illegible] ± [illegible]	[illegible]3 ± [illegible]	[illegible]84 ± 16	200 ± 9	1[illegible] ± 7

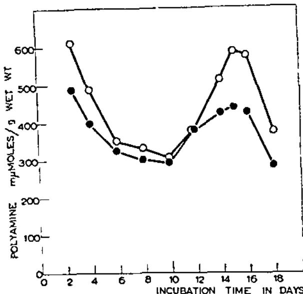


Fig 8 Spermidine and spermine content of the whole chick embryo at various stages of development expressed as mμmoles per g of wet weight
○ = *spermidine* ● = *spermine*

The mean contents of spermidine and spermine as mμmoles per 100 mg of dry weight in relation to the embryo age are presented in Fig 9 With increasing age of the embryos the content of spermidine fell from the maximum value of 1020 mμmoles on the third day of incubation being only one fifth of this value at the age of 18 days The corresponding contents of spermine were 813 and 152 mμmoles respectively However between the ages of 10 and 14 days the content of spermidine per unit of dry weight remained fairly constant indicating as rapid an increase in the polyamine content as in the dry weight The spermine content remained constant between the ages of 10 and 12 days

The total spermidine and spermine contents as μmoles per embryo are plotted against the age of the embryos in Fig 10 The amounts of both polyamines increased steadily up to the age of 16 days In the 16 day embryos the total amount of spermidine was about 8 5 μmoles whereas only 7 4 μmoles was obtained from 18 day embryos A similar decrease was also found in the total amount of spermine after the age of 16 days

grouped according to the age of the embryos The mean contents ± S D (standard deviation) are expressed both as mμmoles per g of wet weight and per 100 mg of dry weight Up to the age of 10 days the values were obtained from pooled samples Table 9 includes a further three groups which have been injected one to three times with 200 μl of 0 9 per cent sodium chloride, as treatment with sodium chloride has been found to have no effect on the polyamine contents

The polyamine contents of embryos belonging to the same incubation batch and age group deviate relatively little from the mean, the standard deviation being with few exceptions about ± 5 per cent Some of the greatest deviations are due to differences in embryo weights On the other hand the degree of correlation between embryo weight and polyamine concentration is not consistent In general, the *dispersion is smaller if the polyamine contents are calculated per unit* of dry weight than per unit of wet weight

As indicated in Table 9 (incubation series 23a b) homogenization in 0 1 or 0 01 N HCl gave essentially the same recoveries The slightly lower values of the relative dry weight and polyamine contents per g of wet weight obtained with 0 01 N HCl were mainly due to a single low value in this group

The polyamine contents of embryos of the same age but from different incubation batches agree fairly well Therefore groups of the same age have been combined and the mean values ± S D have also been calculated from these combined groups In the following, attention is paid to changes in these mean polyamine contents during embryonic development

The mean contents of spermidine and spermine expressed as mμmoles per g of wet weight in relation to embryo age are presented in Fig 8 It can be seen that the changes in the concentrations of these two amines are fairly parallel The highest concentrations *611 mμmoles* of spermidine and *488 mμmoles of spermine per g were obtained* on the third day of incubation The concentrations of both polyamines then gradually fell to a low level, about 300 mμmoles of each polyamine in the 8 to 10 day old embryos There followed a rapid increase in the polyamine contents which reached its maximum about 600 mμmoles of spermidine and 430 mμmoles of spermine on the 16th day of incubation After this stage there was a sharp decrease in the polyamine contents as only about 370 mμmoles of spermidine and 280 mμmoles of spermine were detected in the 18 day embryos

The molar ratio spermidine/spermine varied between 1 0 and 1 35 during the course of development It gradually decreased from a value of 1 25 on the third day to about 1 on the 12th day after which it increased being above 1 3 from the 15th to the 18th day

Contents of Spermidine and Spermine in Various Tissues of the Embryo and in Extra embryonic Parts

The contents of spermidine and spermine in brain heart, small intestine liver and skeletal muscle of 18 day embryos are presented in Table 10 The values were obtained from samples consisting of the pooled organs of 6 to 10 embryos

Table 10 Spermidine and spermine content in some tissues of 18-day-old chick embryos Polyamine content expressed as mµ moles per g of wet weight and per 100 mg of dry weight relative dry weight as mg per g of wet weight For determination 6 to 10 organs or tissue samples were pooled Spd = spermidine Sp = spermine

Tissue	Relat dry weight	Polyamine content				Molar ratio Spd/Sp
		mµM/g w wt		mµM/100 mg d.wt		
		Spd	Sp	Spd	Sp	
Brain	132	333	462	253	351	0 72
Heart	137	257	548	187	400	0 47
Intestine	133	539	612	406	461	0 88
Liver	135	878	5	651	558	1 17
Muscl (th gh)	14	456	363	315	250	1 26

There are marked differences between the polyamine contents of the different tissues The highest spermidine content 878 mµmoles per g of fresh tissue was obtained from the liver whereas less than one third of the liver concentration was found in the heart The liver was also rich in spermine containing 752 mµmoles per g whereas only 363 mµmoles per g was obtained from the skeletal muscle

The molar ratio spermidine/spermine also varied markedly from tissue to tissue Thus the molar concentration of spermidine in the heart was only one half of the spermine concentration whereas in skeletal muscle and liver its molar concentration exceeded that of spermine

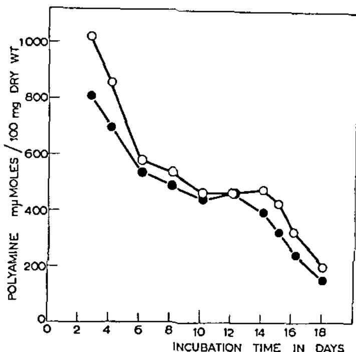


Fig 9 Spermidine and spermine content of the whole chick embryo at differen stages of development expressed as mμmoles per 100 mg of dry weight
○ = *spermidine* ● = *spermine*

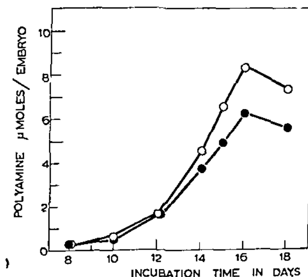


Fig 10 Total spermidine and spermine content (μmoles/embryo) of the chick embryo in relation to age
○ = *spermidine* ● = *spermine*

day earlier the similarity of the ribonucleic acid curve to that of the polyamines is obvious The nucleic acid content per unit of dry weight (REDDY LOMBARDO and CERECEDO 1952 MAPRIAN HUGHES and WERBA 1956) also shows changes similar to those occurring in the polyamines Thus it can be stated that the polyamines and ribo nucleic acid show similar changes during embryonic development but the importance of this correlation as well as the causal relation *is not possible to evaluate at present*

The total nitrogen content expressed as a percentage of the wet weight (LEVY and PALMER 1940) shows minor changes during develop ment However a peak can be observed on the 2nd day and another on the 17th day *i e* somewhat later than in the polyamines Per unit of dry weight the total nitrogen varies within a range of 10 to 12 per cent of the dry weight (KIVIRIKKO 1963) The changes in free amino nitrogen (KIVIRIKKO 1963) from the 10th to the 18th day are fairly similar to those of the polyamines It is evident that these peaks observed at the age of 14 to 16 days are correlated with general changes in the nitrogen metabolism of the embryo Thus for example of the total material that is resorbed daily protein constitutes the largest proportion at this stage (NEEDHAM 1942 p 62) Certain enzymes from whole chick embryo extracts for example dipeptidase (LEVY and PALMER 1940) and aminopeptidase (LEVY and PALMER 1943) show two peaks of activity one on the 2nd day and the other on the 14th to 16th days some others for example glutamic dehydrogenase (SOLOMON 1957) show a similar peak on the 15th to 16th day

The occurrence of spermidine and spermine in the chick embryo in relatively large amounts as noted in the present study may also be taken as evidence that the polyamines are connected in some way with growth and protein synthesis Large amounts of polyamines especially of spermidine were also found in newborn rat liver where the concentration of spermidine was several times that of spermine (ROSENTHAL and TABOR 1956) Further support for this view was afforded by the observations that the tissues in which protein syn thesis is rapid such as the bone marrow in leukemia (HAMALAINEN 1941) or the liver and pancreas (ROSENTHAL and TABOR 1956) are *especially rich in polyamines* However the possible physiological *significance of these facts* is obscure at the present time

According to the present results there are marked differences between the polyamine contents of *different* tissues of the chick

The polyamine content in the extra embryonic parts was in general much lower that in the embryo. On the 16th day of incubation the *yolk sac contained about 40* mμmoles of spermidine and 90 mμmoles of spermine per g of wet matter, two days later the concentrations of both polyamines were only one half of these values. In amniotic and allantoic fluids both spermidine and spermine were only found in traces ($<$ 5 mμmoles/ml) at different stages of development.

DISCUSSION

The results of the present investigation demonstrate that the *polyamine concentration in the whole chick embryo* is highest at the *early* stage of development. Another peak was found at the age of 15 to 16 days, after which a sharp decrease was observed. The polyamine content per unit of dry weight decreased continuously during development, except at the age of 10 to 14 days, at which time it *remained fairly constant. The changes in both spermidine and spermine* at different stages were fairly parallel.

The results further indicate that the concentrations of spermidine and spermine in whole chick embryos varied within quite a small range in groups of the same age. On the other hand, the changes *in the polyamine contents at different stages are apparently connected* with the changes in metabolism that occur during embryonic development. As stated previously, some recent studies have shown that the polyamines like Mg^{++} are able to stabilize nucleic acids and the nucleic acid containing particles of the cell. In addition, they are in *certain conditions able to stimulate* incorporation of amino acids into protein (see pp. 18—21). These studies suggest that spermidine and spermine are possibly connected in some way with nucleic acid and protein metabolism. It is therefore interesting to compare the changes in the polyamines with those observed in the nucleic acids and nitrogen content during embryonic development.

According to NOVIKOFF and POTTER (1948), the concentration of deoxyribonucleic acid (per cent wet weight) in the whole chick embryo rises from *the 2nd to the 15th day, after which it falls.* In contrast, the concentration of ribonucleic acid has two maximum values, one on the 2nd and the other on the 14th day, after which it falls sharply. Except that the latter maximum is reached about one

day earlier the similarity of the ribonucleic acid curve to that of the polyamines is obvious The nucleic acid content per unit of dry weight (REDDY LOMBARDO and CERECEDO 1952 MARRIAN HUGHES and WERBA 1956) also shows changes similar to those occurring in the polyamines Thus it can be stated that the polyamines and ribonucleic acid show similar changes during embryonic development but the importance of this correlation as well as the causal relation is not possible to evaluate at present

The total nitrogen content expressed as a percentage of the wet weight (LEVY and PALMER 1940) shows minor changes during development However a peak can be observed on the 2nd day and another on the 17th day i e somewhat later than in the polyamines Per unit of dry weight the total nitrogen varies within a range of 10 to 12 per cent of the dry weight (KIVIRIKKO 1963) The changes in free amino nitrogen (KIVIRIKKO 1963) from the 10th to the 18th day are fairly similar to those of the polyamines It is evident that these peaks observed at the age of 14 to 16 days are correlated with general changes in the nitrogen metabolism of the embryo Thus for example of the total material that is resorbed daily protein constitutes the largest proportion at this stage (NEEDHAM 1942 p 62) Certain enzymes from whole chick embryo extracts, for example dipeptidase (LEVY and PALMER 1940) and aminopeptidase (LEVY and PALMER 1943) show two peaks of activity one on the 2nd day and the other on the 14th to 16th days some others for example glutamic dehydrogenase (SOLOMON 1957) show a similar peak on the 15th to 16th day

The occurrence of spermidine and spermine in the chick embryo in relatively large amounts as noted in the present study may also be taken as evidence that the polyamines are connected in some way with growth and protein synthesis Large amounts of polyamines especially of spermidine were also found in newborn rat liver where the concentration of spermidine was several times that of spermine (ROSENTHAL and TABOR 1956) Further support for this view was afforded by the observations that the tissues in which protein synthesis is rapid such as the bone marrow in leukemia (HAMALAINEN 1941) or the liver and pancreas (ROSENTHAL and TABOR 1956) are especially rich in polyamines However the possible physiological significance of these facts is obscure at the present time

According to the present results there are marked differences between the polyamine contents of different tissues of the chick

embryo. The highest values were obtained from the liver. They were somewhat lower than the values obtained from livers of mouse, rat or rabbit (ROSENTHAL and TABOR 1956). The molar ratio spermidine/spermine also varied appreciably, being above 1 in the liver and skeletal muscle. It is probable that the molar ratio reflects the metabolic activity of the polyamines in the tissue in question. ROSENTHAL and TABOR (1956) reported that only traces of polyamines were recovered from the liver of the chicken, which they suggested to be due to a high turn over rate. However, some preliminary experiments by the present author (unpublished) seem to indicate that the polyamine content in the chicken liver is of the same order as in chick embryos, but the concentration of spermine is nearly twofold compared with that of spermidine. At present the extent to which degradation of polyamines occurs in the chick embryo is not known. The total content of polyamines per embryo decreased after the age of 16 days. It is therefore likely that this decrease is due to increased degradation, because the contents of the yolk sac and of the amniotic and allantoic fluids were quite small and did not indicate noteworthy excretion of polyamines.

BIOSYNTHESIS OF SPERMIDINE AND SPERMINE IN THE DEVELOPING CHICK EMBRYO

Preliminary Experiments

In preliminary experiments several non labelled compounds which might act as precursors in the biosynthesis of polyamines were injected into eggs in order to demonstrate changes in the polyamine contents of the embryos. These compounds, injected into the air space on the 8th to 10th day of incubation in amounts of 1 to 10 mg, included putrescine (Hoffmann — La Roche), DL ornithine (Fluka) and L methionine (Fluka). However, no consistent changes were found in the polyamine contents.

Effect of Ethionine on the Polyamine Content of Whole Chick Embryos

In micro organisms it has been shown that methionine participates in the biosynthesis of spermidine. Assuming that the mechanism is also the same in the chick embryo, it seemed likely that ethionine

Table 11 *Effect of DL ethionine on the spermidine and spermine contents of whole chick embryos*

Ethionine was injected in 200 μl of 0 9 per cent sodium chloride into the air space controls receiving the same amount of 0 9 per cent sodium chloride Relative dry weight expressed as mg per g wet weight and polyamine contents as mμmoles per g wet weight and per 100 mg dry weight Mean values ± standard deviation

C = controls E = *treated with ethionine* Spd = *spermidine* Sp = *spermine*

Treatment Series I 20 μmoles of ethionine to 12-day old embryos analysed 40 h after injection

Series II 15 μmoles of ethionine twice the first injection at 12 days the second at 13 days Analysed ?4 h after the latter administration

Series III 15 μmoles three times the first injection at 12 days the two others at intervals of 24 h each Analysed 24 h after the last injection

Series		Number of embr	Wt of embryos	Relat dry wt	Polyamine content mμmoles /g wet wt Spd	Sp	/100 mg d wt Spd	Sp	Molar ratio Spd/Sp
I	C	6	8 37 ± 0 4	100 ± 7	04 ± 56	394 ± 40	502 ± 34	393 ± 19	1 28
	E	6	61 * ± 0 38	106 ± 10	550 ± 55	359 ± ?6	519 ± ?5	340 ± ?4	1 53
II	C	7	9 4? ± 0 46	111 ± 3	530 ± 15	438 ± 16	4?6 ± 18	393 ± 19	1 21
	E	7	8 3? ± 0 88	108 ± 5	543 ± 30	381* * ± 14	505 ± 4	354 ± 18	1 43
III	C	6	11 4 ± 1 36	1?9 ± 5	572 ± 24	439 ± 41	437 ± 30	341 ± 38	1 30
	E	7	10 ?8 ± 1 3?	1?9 ± 9	568 ± 18	359 ± ?9	443 ± 3?	280 ± ?	1 58

P < 0 05 ** P < 0 01 *** P < 0 001

as a methionine antagonist might affect the polyamine concentration The effect of ethionine (DL-ethionine L Light & Co Ltd) was studied in three different experimental series 20 to 45 μmoles of ethionine in one to three doses was injected into eggs incubated 12 to 14 days The treatments and results are presented in Table 11

Ethionine retarded the weight gain of the embryos in all the doses used The mean wet weight of the embryos treated with ethionine

was about 90 per cent of the controls The difference in the first two series was also statistically significant ($P < 0.01$ and < 0.05 respectively) In contrast no noteworthy changes could be found in the relative dry weights

The concentration of spermidine was only slightly affected by ethionine In the first two series the spermidine content was even higher in the ethionine treated groups than in the controls However, owing to the difference in the weights of the embryos the total amount of spermidine per embryo was 1 to 9 per cent smaller in the ethionine treated groups than in the controls

In contrast to spermidine, the concentration of spermine was significantly lower in all the groups that received ethionine compared with the controls The contents of spermine, expressed per unit of wet weight, were in the first series 9 in the second 13 and in the third series 18 per cent lower in the ethionine treated embryos The total amount of spermine per embryo was 17 to 25 per cent smaller in the embryos treated with ethionine Most of the changes in spermine were also statistically significant

The molar ratio spermidine/spermine also clearly reflects the effect of ethionine on the polyamines which was to cause an increase of 18 to 22 per cent in the molar ratio This increase was chiefly due to the decrease in the spermine content and to a lesser extent to the increase in the spermidine content

Incorporation Studies with ^{14}C Labelled Compounds

In the following experiments 1 4 ^{14}C putrescine 2 ^{14}C DL ornithine and 2 ^{14}C DL methionine were used to study the biosynthesis of polyamines in the developing chick embryo Radioactivity was found in both spermidine and spermine after administration of all these compounds In contrast no radioactivity could be detected in the polyamines after administration of uniformly labelled ^{14}C glucose or uniformly labelled ^{14}C proline (both from The Radiochemical Centre Amersham dose 2 μC/egg) In addition the effect of ethionine on the methionine incorporation was studied Some of these results have been presented in a preliminary note (RAINA 1962b) and are discussed in greater detail in the following section

In these experiments 5 to 10 μC of 1 4 ^{14}C putrescine was injected into eggs on the 9th day of incubation At appropriate time intervals after the injection the embryos were analysed one at a time The specific and total activities of the polyamines are presented in Table 12 and Fig 11

Table 12 Incorporation of radioactivity into polyamines in the chick embryo after injection of 1 4 ^{14}C putrescine on the 9th day of development Specific activity counts per minute per μmole of polyamine One embryo analysed at a time

Time after injection h	Dose μC	Specific activity	
		Spermidine	Spermine
5	5	17 300	1 [illegible]00
15	5	59 300	19 800
[illegible]	[illegible]	0 400	5 [illegible]00
30	5	54 700	8 000
60	[illegible]	27 600	[illegible]9 600
140	5	1 800	4 500
27	10	8 [illegible]00	4[illegible] [illegible]00

The specific activity of spermidine (cpm/μmole) 5 h after injection of 5 μC of putrescine was 17 300 cpm/μmole and increased steadily for about twenty hours The specific activity then began to decrease being only 1800 cpm 140 hours (6 days) after injection

The specific activity of the spermine fraction increased more slowly than that of spermidine and at its maximum was less than half that observed in spermidine 5 hours after administration the radioactivity in the spermine fraction barely exceeded that of the background 15 hours after injection the specific activity of spermine was 19 800 and at 22 5 hours 25 700 cpm/μmole then remaining fairly constant up to at least 60 hours 6 days after administration the specific activity was only 4 500 cpm

The corresponding changes in the total activities of spermidine and spermine (cpm/embryo) are presented in Fig 11 Spermidine reached a total activity of about 37 000 cpm/embryo 22 5 to 30 hours after the injection and then slowly decreased being 9 000 cpm 6 days

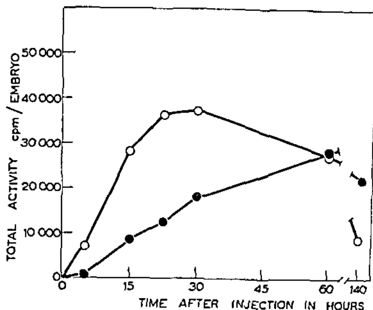
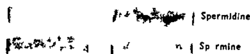


Fig 11 Incorporation of radioactivity into spermidine and spermine in the chick embryo after injection of 5 μC of 1 4 ¹⁴C putrescine on the 9th day of development Total activity as counts per minute per embryo

○ = spermidine ● = spermine



A B

Fig 12 Autoradiogram (B) obtained from extract of a chick embryo treated with 10 μC of ¹⁴C putrescine and analysed 27 h later Paper electrophoretic strip stained with amido black and then exposed to film (Kodak »Industrex« Type D) Exposure time 30 days A duplicate strip stained with amido black

after administration The total activity of spermine increased up to at least 60 hours being at this time approximately the same as that of spermidine 6 days after the injection the total activity was still 22 300 cpm or about 2 5 times that of spermidine It can be further noted that at 60 hours a total activity of about 56 000 cpm was recovered in the polyamines whereas only 31 000 cpm was detected 6 days after administration Of the total radioactivity injected per egg about 1 per cent could be detected in the polyamines 60 hours after administration

The radioactivity of the putrescine fraction gradually decreased and was about 3 per cent of that in the polyamines 60 hours after administration of 5 μC of putrescine Thus the administered putrescine is largely metabolized by some other pathway than via the polyamines

The radioactivity of spermidine and spermine 27 hours after injection of 10 μC of ^{14}C putrescine is also shown by the autoradio graphic technique (Fig 12) In the electropherogram stained with amido black the only active fractions were the polyamines indicating the washing off of other fractions for example putrescine which was highly active in the unstained strips

Incorporation of 2 ^{14}C DL-Ornithine

The specific activities of the polyamines (cpm/μmole) after ad ministration of 1 μC of 2 ^{14}C DL ornithine on the 9th day of incubation are presented in Table 13 The incorporation of radioactivity after ornithine was quite similar to that after putrescine However the

Table 13 Incorporation of radioactivity into polyamines in the chick embryo after administration of ^{14}C DL ornithine on the 9th day of development Specific activity counts per minute per μmole of polyamine One embryo analysed at a time

Time after injection h	Dose μC	Specific activity	
		Spermidine	Spermine
[illegible]	1	[illegible] 140	00
15	1	[illegible]000	1 600
30	1	6 600	3 400
60	1	16 [illegible] 0	2 080
30	5	61 [illegible] 00	3 [illegible] 800

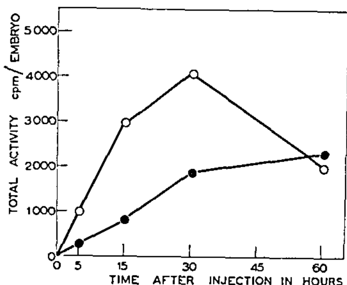


Fig 13 Incorporation of radioactivity into polyamines in the chick embryo after injection of 1 μC of 2 ¹⁴C DL ornithine on the 9th day of development Total activity as counts per minute per embryo
○ = *spermidine* ● = *spermine*

specific activities were only about one tenth of those obtained with putrescine, partly because the dose used was smaller With a dose of 5 μC, the specific activities were of the same order as those obtained with 5 μC of putrescine

The total activities (cpm/embryo) of spermidine and spermine obtained with 1 μC of ornithine are presented in Fig 13 The curves are fairly similar to those for putrescine showing that there was a more rapid incorporation of radioactivity into spermidine in the beginning whereas 60 hours after injection the total activity of spermine exceeded that of spermidine

In addition to spermidine and spermine a weak more rapidly moving fraction could be detected as a rule in electropherograms of chick embryo extracts stained with ninhydrin After administration of labelled ornithine this fraction contained radioactivity showing a maximum total activity about 5 times that of spermidine 5 hours after injection of ornithine This fraction had the same mobility as putrescine in paper electrophoresis (cf Fig 5) and in two dimensional paper chromatography The identity of this compound with putrescine although likely has not yet been strictly confirmed

In these experiments 5 μC of 2-^{14}C DL methionine was administered to eggs on the 10th day of incubation Incorporation of radioactivity into the polyamines is seen in Table 14 and Fig 14

The specific activity (cpm/μmole Table 14) of spermidine increased rapidly and reached its maximum about 59 000 cpm 15 hours after

Table 14 Incorporation of radioactivity into polyamines in the chick embryo after administration of 5 μC of 2-^{14}C DL methionine on the 10th day of development Specific activity counts per minute per μmole of polyamine Each value represents the specific activity obtained from a single embryo

Time after injection h	Specific activity	
	Spermidine	Spermine
5	30 600	19 000
15	59 100	45 600
30	49 [illegible]00	63 900
60	31 100	60 100
	34 600	66 500

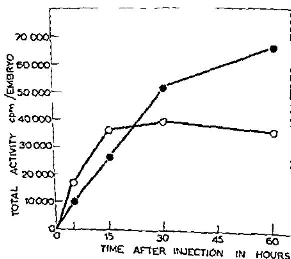


Fig 14 Incorporation of radioactivity into polyamines in the chick embryo after injection of 5 μC of 2-^{14}C DL methionine Total activity as counts per minute per embryo ○ = spermidine ● = spermine

administration and then gradually decreased Except that the peak was reached earlier, the curve of activity is quite similar to that obtained with putrescine In contrast, incorporation of radioactivity into spermine after administration of methionine was more rapid than after putrescine As little as 5 hours after treatment the specific activity of spermine was 19 000 cpm and exceeded the maximum activity of spermidine about 30 h after injection

The total activity (cpm/embryo Fig 14) of spermidine reached its maximum between 15 and 30 hours after injection and showed only a slight decrease at 60 hours The total activity of spermine gradually increased up to at least 60 hours at which time it was 65 000 cpm or nearly twofold that of spermidine Of the total radioactivity injected per egg about 2 8 per cent was recovered in the polyamines of the embryo 60 hours after administration

Effect of Ethionine on the Incorporation of 2 ¹⁴C Methionine

In these experiments 6 μmoles of DL ethionine was injected into eggs on the 10th day of incubation 5 hours later 2 μC of 2 ^{14}C DL methionine was injected into the eggs treated with ethionine and into those serving as controls Analyses were made 30 hours after the latter injection The results are presented in Table 15

Ethionine caused a retardation in the weight gain similar to that described previously The mean wet weight of the control group was 2 72 g and 2 46 g or 10 per cent lower in the group that received ethionine

Incorporation of radioactivity into polyamines was quite uniform in the embryos of the same group The specific and total activities of spermidine were only slightly affected by ethionine The mean specific activity of spermine was 23 900 cpm/μmole in the control group and 14 per cent lower in the ethionine treated group The mean total activity (cpm/embryo) of spermine was 20 per cent lower in the ethionine treated embryos than in the controls

The results described here are in agreement with the data presented in a previous section They confirm the observation that ethionine in the doses used especially affects the synthesis of spermine and only slightly the synthesis of spermidine

Table 15 *Effect of ethionine on* ^{14}C *methionine incorporation into polyamines in the chick embryo*

6 µmoles of DL ethionine was administered into the air space on the 10th day of incubation in 200 µl 0 9 per cent sodium chloride controls receiving sodium chloride solution only 5 h later 2 µC of 2 ^{14}C DL methionine in 200 µl of sodium chloride solution was administered to each egg Analysis 30 h after the latter injection

Spd = spermidine Sp = spermine

Treatment	No of embryo	Wt of embryo	Specific activity cpm per µmole		Total activity cpm per embryo	
			Spd	*Sp*	*Spd*	*Sp*
^{14}C methionine	1	2 8	19 100	23 800	17 400	0 900
	2	2 69	17 600	23 000	18 800	19 800
	3	2 61	19 500	25 000	17 [illegible]00	21 000
	4	2 5	19 100	23 900	17 800	21 400
	Mean	[illegible]	18 800	23 900	17 400	20 800
Ethionine + ^{14}C methionine	1	2 33	18 100	20 500	15 600	15 800
	2	[illegible]	19 700	21 500	1[illegible] 500	15 300
	3	73	18 000	19 700	17 400	17 400
	4	2 48	19 500	0 800	18 [illegible]00	17 800
	Mean	2 46	18 600	20 600	16 700	16 600

DISCUSSION

The results of the present experiments with labelled compounds demonstrate that putrescine ornithine and methionine can act as precursors in the biosynthesis of spermidine and spermine in the developing chick embryo whereas radioactivity was not incorporated into the polyamines after administration of labelled glucose or labelled proline Ethionine reduces the *concentration of spermine in the* whole embryo and also reduces the incorporation of radioactivity into spermine after administration of labelled methionine

Experiments with micro organisms have shown that putrescine and ornithine are incorporated into spermidine and spermine being the source of the four carbon chain of the polyamines (TABOR ROSENTHAL and TABOR 1956 1958) It has further been shown by GREENE (1957) that the source of *the three* carbon chain of spermidine is methionine Experiments with partially purified enzyme systems from

administration and then gradually decreased Except that the peak was reached earlier, the curve of activity is quite similar to that obtained with putrescine In contrast incorporation of radioactivity into spermine after administration of methionine was more rapid than after putrescine As little as 5 hours after treatment the specific activity of spermine was 19 000 cpm and exceeded the maximum activity of spermidine about 30 h after injection

The total activity (cpm/embryo, Fig 14) of spermidine reached its maximum between 15 and 30 hours after injection and showed only a slight decrease at 60 hours The total activity of spermine gradually increased up to at least 60 hours at which time it was 65 000 cpm or nearly twofold that of spermidine Of the total radioactivity injected per egg about 2 8 per cent was recovered in the polyamines of the embryo 60 hours after administration

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The results described here are in agreement with the data presented in a previous section They confirm the observation that ethionine in the doses used especially affects the synthesis of spermine and only slightly the synthesis of spermidine

probable that the polyamines in adult tissues are metabolically more or less inert and their synthesis therefore difficult to demonstrate In contrast as stated in the present study synthesis in developing embryos is quite rapid It seems likely that by using embryonic tissues it will also be possible to demonstrate the enzymic synthesis of polyamines in animal tissues

Of the compounds which are incorporated into the polyamines putrescine is widely distributed in micro organisms (HERBST WEAVER and KEISTER 1958) but not until recent years has it been shown that small amounts of putrescine also occur in animal tissues *e g* in liver and pancreas (FISCHER and BOHN 1957b WEAVER and HERBST 1958) In the present study a weak ninhydrin positive fraction could repeatedly be detected in extracts of chick embryos This showed a mobility similar to putrescine in paper electrophoresis and paper chromatography It is therefore possible that in animal tissues also putrescine is a natural precursor in the biosynthesis of polyamines The above mentioned fraction from chick embryo extracts contained radioactivity after administration of 2 ^{14}C labelled ornithine and therefore it is possible that incorporation of ornithine into polyamines occurs *via* putrescine

In experiments with ethionine a retardation in weight gain was observed in all the doses used This effect of ethionine on the rapidly growing organism is at least partly due to inhibition of protein synthesis (*e g* SIMPSON FARBER and TARVER 1950 FARBER and CORBAN 1958) Similar effects have also been obtained with several other methionine homologues (CESTARI *et al* 1962) In contrast to the results of CESTARI *et al* no noteworthy changes were found in the relative dry weights in the present study which is probably due to the smaller doses of ethionine used

The effect of ethionine on the contents of the polyamines was somewhat unexpected While the concentration of spermidine as well as the incorporation of labelled methionine into spermidine were only slightly affected or even increased by ethionine the concentration of spermine was significantly lower in the ethionine treated groups Ethionine also reduced the incorporation of labelled methionine into spermine Because methionine participates in the biosynthesis of polyamines these effects of ethionine may be regarded as antagonistic effects of ethionine on methionine metabolism In recent years evidence has been presented that the inhibiting effect of

Escherichia coli have confirmed these results and shown that the enzymic synthesis of spermidine from putrescine and methionine involves three different steps (cf p 16), viz formation of S adenosyl methionine, which then, after decarboxylation, serves as the donor of the propylamine moiety to putrescine (TABOR, ROSENTHAL and TABOR 1958, TABOR 1962a,b) Up to the present time attempts at the enzymic synthesis of spermine have not met with success but the mechanism has been suggested to be comparable with that described for spermidine (TABOR TABOR and ROSENTHAL 1961) No data have been presented which would indicate incorporation of methionine into spermine in whole bacterial cells

The results of the present study suggest that the mechanism of biosynthesis of the polyamines in the chick embryo is comparable with that in micro organisms Incorporation of radioactivity into spermidine after administration of labelled putrescine ornithine and methionine was fairly rapid and began without any clear lag period In contrast, at 5 hours after injection of labelled putrescine or ornithine, the radioactivity in the spermine fraction barely exceeded that of the background The total activity of spermine then increased during a period of at least 60 hours while the total activity of spermidine began to decrease after only 30 hours It is therefore likely that spermidine is a precursor of spermine Further support for this assumption is given by the finding that after administration of labelled methionine which is the source of the three carbon chain of the polyamines the activities of both spermidine and spermine were from the beginning more or less parallel indicating that the labelled compound was incorporated simultaneously into both polyamines in roughly equivalent amounts On the other hand TABOR (1962b) has reported that *in vitro* systems containing purified propylamine transferase from *E coli* spermidine could not substitute for putrescine as an acceptor of the propylamine moiety However it seems evident that both the propylamine moieties of spermine are derived from methionine since at 60 hours the specific and total activities of spermine were about twofold those of spermidine

Until recently the biosynthesis of spermidine and spermine in animal tissues has been quite obscure A small incorporation of ^{14}C ^{15}N putrescine mainly into spermidine was obtained in minced rat prostate but no incorporation was found in minced liver muscle spleen or kidney (TABOR ROSENTHAL and TABOR 1956) It appears

probable that the polyamines in adult tissues are metabolically more or less inert and their synthesis therefore difficult to demonstrate. In contrast, as stated in the present study, synthesis in developing embryos is quite rapid. It seems likely that by using embryonic tissues it will also be possible to demonstrate the enzymic synthesis of polyamines in animal tissues.

Of the compounds which are incorporated into the polyamines putrescine is widely distributed in micro-organisms (HERBST, WEAVER and KEISTER 1958) but not until recent years has it been shown that small amounts of putrescine also occur in animal tissues, *e.g.* in liver and pancreas (FISCHER and BOHN 1957b, WEAVER and HERBST 1958). In the present study a weak ninhydrin-positive fraction could repeatedly be detected in extracts of chick embryos. This showed a mobility similar to putrescine in paper electrophoresis and paper chromatography. It is therefore possible that in animal tissues also putrescine is a natural precursor in the biosynthesis of polyamines. The above-mentioned fraction from chick embryo extracts contained radioactivity after administration of 2-^{14}C labelled ornithine, and therefore it is possible that incorporation of ornithine into polyamines occurs *via* putrescine.

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The effect of ethionine on the contents of the polyamines was somewhat unexpected. While the concentration of spermidine as well as the incorporation of labelled methionine into spermidine were only slightly affected, or even increased by ethionine, the concentration of spermine was significantly lower in the ethionine-treated groups. Ethionine also reduced the incorporation of labelled methionine into spermine. Because methionine participates in the biosynthesis of polyamines these effects of ethionine may be regarded as antagonistic effects of ethionine on methionine metabolism. In recent years evidence has been presented that the inhibiting effect of

ethionine on protein synthesis is not a direct inhibition of methionine metabolism, but rather due to the adenine trapping effect of ethionine It has been shown that the S adenosyl derivatives of methionine and ethionine are formed at almost equal rates in rat liver, but that the adenine moiety of S adenosylethionine is much less available than that of S adenosylmethionine (MODY *et al* 1963) Thus ethionine causes a decrease in the tissue adenosine triphosphate concentration (SHULL 1962 BARTELS and HOHORST 1963) Administration of adenine or adenosine triphosphate protects against the inhibition of protein synthesis induced by ethionine (VILLA TREVINO and FARBER 1962 VILLA TREVINO, SHULL and FARBER 1963) It is probable that the mechanism by which ethionine caused a decrease in the spermine content is comparable with that described above This however does not explain the difference between spermidine and spermine in this respect, presuming that the mechanisms of synthesis of these amines are comparable This difference, as well as the extent if any, to which ethionine can act as a precursor in the synthesis of polyamines, needs further elucidation

Degradation of polyamines in the chick embryo has not been studied in the present work Some indirect evidence, however indicates that degradation does occur The isotope experiments with labelled putrescine showed that of the total activity detected in the polyamines at 60 hours after administration, about one half was recovered at 140 hours As discussed previously the decrease in the total content of polyamines per embryo at a late stage of development may also indicate increased degradation

GENERAL SUMMARY

The objects of the present investigation were to develop a quantitative method for the determination of the spermidine and spermine contents of tissues and to study the metabolism of these polyamines in the developing chick embryo with special reference to their biosynthesis

The quantitative determination of spermidine and spermine in tissues consisted of the following steps 1) partial purification by extraction of amines from alkaline protein free tissue extracts into *n* butanol 2) subsequent separation of amines by paper electrophoresis and 3) quantitative determination of spermidine and spermine with amido black The various steps have been studied separately and the optimum conditions are given

The present method for quantitative determination of spermidine and spermine with amido black is based on complex formation between amido black and polyamines two moles of amido black per mole of polyamine a complex which is very sparingly soluble in a methanol–acetic acid mixture but dissolves readily in alkaline solution The present method consisted of spraying the electrophoretic paper strips with amido black solution drying them and washing off the extra colour The coloured fractions were then cut off eluted in alkaline solution and determined spectrophotometrically at 615 mμ The method has proved to be sensitive reproducible and specific for spermidine and spermine allowing an accurate determination of quantities of these amines as small as 5 mμmoles per strip Duplicate determinations deviated from the mean by less than 2 to 3 per cent Absorption was rectilinear up to at least 50 mμmoles of polyamine per strip

The concentrations of spermidine and spermine in the whole chick embryo were determined at different stages of development from the third day of incubation The highest values 611 mμmoles of spermidine and 488 mμmoles of spermine per g of wet weight were obtained on the third day of incubation The polyamine concentration

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gradually decreased to a low level at the age of 10 days and then increased, reaching a peak at the age of 15 to 16 days A sharp decrease was observed after this stage Per unit of dry weight the polyamine contents decreased during development from values of 1020 mμmoles of spermidine and 813 mμmoles of spermine per 100 mg of dry weight on the third day of incubation to 200 mμmoles of spermidine and 152 mμmoles of spermine per 100 mg of dry weight in the 18 day old embryos

Great differences were found between the polyamine contents of different tissues analysed at the age of 18 days The highest values, 878 mμmoles of spermidine and 752 mμmoles of spermine were obtained from the liver The molar ratio spermidine/spermine also showed marked differences from tissue to tissue The lowest value 0 47, was obtained from the heart and the highest 1 26 from the skeletal muscle The polyamine contents of the extra embryonic parts were much lower than those of the embryo

Studies with ^{14}C labelled compounds showed that radioactivity was incorporated into both spermidine and spermine after administration of 1,4 ^{14}C putrescine 2 ^{14}C DL ornithine or 2 ^{14}C DL methionine into the egg but not after administration of uniformly labelled ^{14}C glucose or ^{14}C proline Measurements of radioactivity were carried out in a liquid scintillation counter directly from the paper Both the specific and total activities (per embryo) were determined

After administration of labelled putrescine or ornithine incorporation of radioactivity into spermidine was quite rapid and reached a maximum within 30 hours whereas incorporation into spermine was slower showed a clear latent period at the beginning and then increased for at least 60 hours After administration of labelled methionine *incorporation of radioactivity into* spermidine was fairly similar to that obtained with putrescine and ornithine whereas incorporation into spermine was more rapid and began without any clear latency 60 hours after the injection of methionine the total activity of spermine per embryo was about twofold compared with spermidine

Of the total radioactivity injected per egg about 1 per cent after injection of putrescine and 2 8 per cent after injection of methionine was recovered in the polyamines 60 hours after administration

Ethionine in amounts totalling 20 to 45 μmoles per egg retarded the weight gain of the embryos by about 10 per cent The concentration of spermidine was only slightly affected or even increased by

ethionine treatment whereas a decrease of 9 to 18 per cent was observed in the concentration of spermine Ethionine also reduced incorporation of radioactivity into spermine after injection of labelled methionine but had no noteworthy effect on incorporation into spermidine

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PREFACE

The present work was carried out at the Department of Medical Chemistry University of Helsinki I am very much indebted to the former Head of this Department the late Professor P E Simola M D Ph D for suggesting to me the subject of this investigation and for his kindness in placing the facilities of the Department at my disposal

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Kari Kivirikko

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CONTENTS

INTRODUCTION

Hydroxyproline is an amino acid found in the animal organism in specific relation to the connective tissue Practically all protein bound hydroxy proline occurs in collagen Apart from this only very small amounts are present in elastin Therefore it has been generally assumed that all hydroxy proline is derived from collagen

Collagen is the main fibrous protein of the connective tissue and the most abundant protein in the body It has a characteristic amino acid composition for the imino acids proline and hydroxyproline together make up almost one-third and the amino acid glycine another third of the collagen molecule Special interest attaches to the two hydroxy acids hydroxyproline and hydroxylysine which are almost specific to collagen It is further known that they are not derived from free hydroxyproline or hydroxylysine but from proline and lysine which are hydroxylated during the synthesis of collagen

Besides its amino acid composition collagen has other characteristic features such as its appearance under the electron microscope its X ray diffraction pattern and physicochemical properties These various characteristic features have made it possible to study collagen by several different methods Investigations during the past decade have indicated that collagen fibres are composed of rod like macromolecules called tropocollagen which are synthesized within the fibroblasts and removed from there into the extracellular space where they combine to form the collagen fibres When connective tissue is extracted with various organic acids or salt solutions the collagen molecules are dissolved to varying extents depending on the sol vent used The significance of these macromolecular hydroxyproline-containing fractions in the normal metabolism of collagen and in conditions with altered metabolism has been the subject of several investigations during the last decade Furthermore during recent years increasing attention has been paid to the free and peptide bound hydroxyproline present in the tissues and biological fluids with a view to explaining the role of these low molecular fractions in the metabolism of collagen

REVIEW OF THE LITERATURE

RELATION OF VARIOUS HYDROXYPROLINE CONTAINING FRACTIONS TO THE METABOLISM OF COLLAGEN

Although collagen has long been regarded as an insoluble protein it has been known since the beginning of this century that part of the rat tail tendon collagen will dissolve in dilute acetic acid (ZACHARIADÈS 1900) In a series of investigations NAGEOTTE (e g 1927 1930 1933) studied the solubilisation of rat tail tendon collagen in dilute acetic acid and the formation of fibrils from this solution In 1934 NAGEOTTE and GUYON found that the amount of collagen that could be dissolved in acetic acid decreased with age

V N OREHOVIČ and co workers (e g OREHOVIČ TUSTANOVSKIJ OREHOVIČ and PLOTNIKOVA 1948 OREHOVIČ 1953 OREHOVIČ and ŠPIKITER 1957 OREHOVIČ and ŠPIKITER 1958) studied the properties of a fraction that can be extracted with acid citrate buffer They suggested that this fraction was a precursor of the insoluble collagen and called it procollagen Later it was found that collagen could also be extracted from connective tissue with alkaline phosphate buffer (HIGHBERGER GROSS and SCHMITT 1951) and neutral salt solutions of varying ionic strengths (GROSS HIGHBERGER and SCHMITT 1955) It was shown that the fractions soluble in alkaline phosphate buffer called alkali soluble collagen (HARKNESS MARKO MUIR and NEUBERGER 1954) 0 2 M sodium chloride (JACKSON 1957) and 0 45 M sodium chloride (GROSS 1958 b) represented even earlier stages in the metabolism of collagen than the citrate soluble fraction In addition a water soluble collagen fraction was found to be present in the granuloma induced by carrageenin injection and comprised the greater part of the 0 2 M sodium chloride soluble collagen formed during the early stages of development of the granuloma (JACKSON 1957)

An explanation for the significance of the various soluble collagen fractions in the metabolism of collagen was proposed by JACKSON (1958) GROSS (1959 a) JACKSON (1959) GREEN and LOWTHER (1959) and JACKSON and BENTLEY (1960) Collagen fibres are built up from rod like macromolecules called tropocollagen that have a weight of 360 000 and dimensions of about 14 × 2 800 Å They consist of three polypeptide chains helically coiled upon themselves (see also SCHMITT 1959 HODGE and SCHMITT 1961) These molecules are synthesized within fibroblasts from which they escape into the extracellular space and there aggregate to form collagen fibres Dilute salt solutions (0 14 M NaCl) extract the most recently synthesized molecules which are partly intracellular partly already extracellular but aggregated only very

Since the discovery that cortisone has a therapeutic effect on the collagen diseases, the mechanism by which cortisone acts on connective tissue has been studied in a number of investigations. It has been found that cortisone, at least in large doses, inhibits the metabolism of connective tissue and the formation of new collagen. The mechanism of this inhibition, however, is not known.

Although the problems of connective tissue have been discussed in several reviews and symposia, the rapid advances in the chemistry of collagen make it worth while to introduce the present investigation with a brief review of the literature concerning the metabolic aspects of collagen. This review is mainly concerned with papers that have appeared during the last few years, but the most important of the older publications are also mentioned. For more extensive references to the literature concerning collagen the reader is referred to the proceedings of a conference, edited by STAINSBY (1958), and to the extensive review by HARKNESS (1961) on the biological functions of collagen. Regarding physicochemical and structural aspects, reference should further be made to the articles of HODGE and SCHMITT (1961) and RICH and CRICK (1961). The problems associated with connective tissue in general have been dealt with in the reviews of JACKSON (1958) and SMILEY and ZIFF (1962), for instance, and in the reports of connective tissue symposia edited by TUNBRIDGE (1957) and PAGE (1959).

The object of the present investigation was to make a simultaneous study of the various low-molecular and macromolecular hydroxyproline-containing fractions during the formation of collagen in developing chick embryos. In addition, the effect of the inhibition of collagen formation by cortisone on the proportions of these hydroxyproline-containing fractions was investigated.

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RELATION OF VARIOUS HYDROXYPROLINE CONTAINING FRACTIONS TO THE METABOLISM OF COLLAGEN

Although collagen has long been regarded as an insoluble protein it has been known since the beginning of this century that part of the rat tail tendon collagen will dissolve in dilute acetic acid (ZACHARIADÈS 1900) In a series of investigations NAGEOTTE (e g 1927 1930 1933) studied the solubilisation of rat tail tendon collagen in dilute acetic acid and the formation of fibrils from this solution In 1934 NAGEOTTE and GUYON found that the amount of collagen that could be dissolved in acetic acid decreased with age

V N OREHOVIČ and co workers (e g OREHOVIC TUSTANOVSKIJ OREHOVIČ and PLOTNIKOVA 1948 OREHOVIČ 1953 OREHOVIC and ŠPIKITER 1957 OREHOVIČ and ŠPIKITER 1958) studied the properties of a fraction that can be extracted with acid citrate buffer They suggested that this fraction was a precursor of the insoluble collagen and called it procollagen Later it was found that collagen could also be extracted from connective tissue with alkaline phosphate buffer (HIGHBERGER GROSS and SCHMITT 1951) and neutral salt solutions of varying ionic strengths (GROSS HIGHBERGER and SCHMITT 1955) It was shown that the fractions soluble in alkaline phosphate buffer called alkali soluble collagen (HARKNESS MARKO MUIR and NEUBERGER 1954) 0 2 M sodium chloride (JACKSON 1957) and 0 45 M sodium chloride (GROSS 1958 b) represented even earlier stages in the metabolism of collagen than the citrate soluble fraction In addition a water soluble collagen fraction was found to be present in the granuloma induced by carrageenin injection and comprised the greater part of the 0 2 M sodium chloride soluble collagen formed during the early stages of development of the granuloma (JACKSON 1957)

An explanation for the significance of the various soluble collagen fractions in the metabolism of collagen was proposed by JACKSON (1958) GROSS (1959 a) JACKSON (1959) GREEN and LOWTHER (1959) and JACKSON and BENTLEY (1960) Collagen fibres are built up from rod like macromolecules called tropocollagen that have a weight of 360 000 and dimensions of about 14 × 2 800 Å They consist of three polypeptide chain helically coiled upon themselves (see also SCHMITT 1959 HODGE and SCHMITT 1961) These molecules are synthesized within fibroblasts from which they escape into the extracellular space and there aggregate to form collagen fibres Dilute salt solutions (0 14 M NaCl) extract the most recently synthesized molecules which are partly intracellular partly already extracellular but aggregated only very

loosely or not at all More concentrated salt solutions extract in addition collagen that has become somewhat more firmly associated with the fibrils Citrate buffers and organic acids are capable of dissolving collagen bound still more firmly to the fibril and finally there remains the insoluble collagen which has the lowest metabolic activity

Quite recently BENTLEY and JACKSON (1963) have reported that there are two peaks in the specific activity of the 0 14 M sodium chloride soluble collagen fraction in the skin and wound granulation tissue of guinea pigs after the administration of ^{14}C glycine ^{3}H glycine and ^{3}H proline They suggested that the newly formed collagen is first extractable with 0 14 M sodium chloride for a short period after which it is temporarily rendered unextractable for a short time and then again rendered extractable The cell surface was regarded as its possible site during the period of unextractability

Further fractions are obtained from the soluble collagen fractions when they are denaturated with urea or certain salts or by mild heating at acidic pH The fractions that are formed are called the α and β components and they can be isolated by such methods as ammonium sulphate fractionation or chromatography on carboxymethyl cellulose columns (e g OREHOVIČ and ŠPIKITER 1958 DOTY and NISHIHARA 1958 OREHOVIČ ŠPIKITER MAZUROV and KUNINA 1960 PIEZ WEISS and LEWIS 1960) It has been found that the rate of incorporation of ^{14}C glycine is greater into the α than the β component of the citrate soluble fraction (OREHOVIČ ŠPIKITER KASAKOVA and MAZUROV 1959) and that the alkali soluble procollagen which is close to the 0 2 M sodium chloride soluble collagen fraction consists solely of α components (MAZUROV and OREHOVIČ 1960) PIEZ LEWIS MARTIN and GROSS (1961) and PIEZ EIGNER and LEWIS (1963) have reported that there are two kinds of both α and β components (α_1 and α_2 β_1 and β_2) The neutral salt soluble collagen consisted mainly of α subunits and the ratio of α_1 to α_2 was close to 2 They suggested that these subunits represent single chains of tropocollagen molecules When the molecule matures and becomes insoluble in sodium chloride but can still be dissolved in acid the chains cross link intramolecularly in pairs which are the β components Probably β_1 is composed of one α_1 and one α_2 chain and β_2 of two α_1 chains A further subfraction of denaturated collagen is γ tropocollagen which probably represents a more mature form than the α and β components (ALTGELT HODGE and SCHMITT 1961)

LOWTHER GREEN and CHAPMAN (1961) have studied the metabolic activity of collagen in different subcellular fractions of carrageenin granulomas After the incubation of granuloma slices with ^{14}C proline the ratio of the specific activities of hydroxyproline in the microsomal mitochondrial and nuclear fractions was [illegible] 2 1 indicating that the primary synthesis of collagen takes place in the microsomes The amino acid composition of this microsomal collagen was similar to that of insoluble collagen (EASTOE 1961) In the intact chick embryo the microsomal collagen likewise showed the highest metabolic activity (PROCKOP PETERKOFSKY and UDENFRIEND 1962) Further support for the view that collagen is synthesized in the microsomes was obtained from the observation that chick embryo microsomes can incorporate ^{14}C proline into hydroxyproline in vitro in the absence of mitochondria (PETERKOFSKY and UDENFRIEND 1961 1962)

The results of the above mentioned experiments indicated that collagen is synthesized by the same ribosomal system as has been postulated for the synthesis of other proteins (see also PROCKOP 1962 a) although it is not known whether the whole

tropocollagen peptide chain is synthesized as a single molecule or whether it consists of more than one building block (GERBER and ALTMAN 1961 PROCKOP 1962 a SNELLMAN 1962) There is one special feature however of the synthesis of collagen which is unique Since the experiments of STETTEN and SCHOENHEIMER (1944) and STETTEN (1949) it has been known that the hydroxyproline of collagen i not derived from free hydroxyproline but from proline that is hydroxylated in bound form during the synthesis of collagen This has ince been confirmed by several investigators (e g SMITH and JACKSON 1957 WOLF and BERGER 1958 GREEN and LOWTHER 1959 PETERKOFSKY and UDENFRIEND 1961) Moreover it has been found that the hydroxylysine of collagen originates from free lysine and not from free hydroxylysine (e g SINEX and VAN SLYKE 1955 PIEZ and LIKINS 1957 VAN SLYKE and SINEX 1958 KAO and BOUCEK 1958 a & b SINEX VAN SLYKE and CHRISTMAN 1959 POPENOE and VAN SLYKE 1962) Only in the work of MITOMA SMITH FRIEDBERG and RAYFORD (1959) was slight incorporation of ^{14}C hydroxyproline into collagen found in the chick embryo but the specific activity of bound hydroxyproline was only one tenth of that observed after the administration of ^{14}C proline The later work of PROCKOP PETERKOFSKY and UDENFRIEND (1962) further indicated that free hydroxyprolin is not an appreciable source of collagen hydroxyproline even in the chick embryo

Several investigations have dealt with the question of whether the hydroxylation occurs after the synthesis of the tropocollagen peptide chain or whether activated proline or proline residues of a precursor peptide are already hydroxylated Some observations indirectly favoured the hypothesis that hydroxylation occurs before the completion of the collagen peptide chain (ROBERTSON HIWETT and HERMAN 1959 GREEN and LOWTHER 1959 UDENFRIEND WEISSBACH and MITOMA 1960 HAUSMANN and NEUMAN 1961 DAUGHADAY and MARIZ 1962 b STONE and MEISTER 1962 PETERKOFSKY and UDENFRIEND 1962) Therefore attempts have been made to demonstrate the presence of activated hydroxyproline or an active peptidic precursor LEVINE (1962) who incubated minced skin of young rats with ^{14}C proline found in the medium a non dialyzable fraction in which the specific activity of hydroxyproline was 8 to 12 times as high as that of proline After treatment of this fraction by autoclaving or at pH 10 or with ribonuclease the radioactivity could be removed by dialysis Therefore he suggested that this fraction represents a highly active peptide collagen precursor possibly bound to ribonucleic acid In addition in chick embryos after the administration of ^{14}C proline MANNER and GOULD (1962) have found small amounts of highly reactive hydroxyproline bound to soluble ribonucleic acid As to the mechanism of hydroxylation of proline recent investigations suggest that it is addition of molecular oxygen that is involved rather than dehydrogenation and addition of water (FUJIMOTO and TAMIYA 1962 EBERT and PROCKOP 1962 HURYCH and CHVAPIL 1962 a PROCKOP KAPLAN and UDENFRIEND 1962 KONNO and TETSUKA 1962)

Since free hydroxyproline is not utilized for the synthesis of collagen the question arises of what role is played by the free and peptide bound hydroxyproline present in the tissues and biological fluids in collagen metabolism

Investigators of a Czechoslovakian group have studied the biological significance of ultrafiltrable hydroxyproline i e free and peptide bound hydroxyproline in a series of works (e g CHVAPIL 1958 CHVAPIL 1959 CHVAPIL and ČMUCHALOVÁ 1960 KOBRLE and CHVAPIL 1961 a & b CHVAPIL and ČMUCHALOVÁ 1961 KOBRLE and CHVAPIL 1962 HURYCH and CHVAPIL 1962 a & b CHVAPIL HOLEČKOVÁ ČMUCHALOVÁ KOBRLE and

HURYCH 1962) These investigations indicated the presence of free and peptide bound hydroxyproline in the tissues of several animals and in the carrageenin granuloma In addition some of these peptides were isolated from pig embryo and identified (KOBRLE and CHVAPIL 1961 a) In the carrageenin granuloma maximum accumulation of ultrafiltrable hydroxyproline was found to occur at the same time as that of deoxyribonucleic acids (cells) and after this stage there was a decrease in the content of ultrafiltrable hydroxyproline and an increase in the content of collagen bound hydroxyproline (CHVAPIL and ČMUCHALOVÁ 1960 1961) The bulk of the hydroxyproline present in the microsomal fraction of the carrageenin granuloma was found to be ultrafiltrable (CHVAPIL HOLEČKOVÁ ČMUCHALOVÁ KOBRLE and HURYCH 1962) It was suggested that free and peptide bound hydroxyproline participate in the synthesis of collagen and are intracellular although their origin by breakdown could not be excluded (CHVAPIL and ČMUCHALOVÁ 1961, CHVAPIL HOLEČKOVÁ ČMUCHALOVÁ KOBRLE and HURYCH 1962) Additional support for this view was obtained from the observations that during the incubation of chick embryo skin with ^{14}C proline the specific activity of ultrafiltrable hydroxyproline was considerably higher than that of neutral salt soluble or insoluble collagen hydroxyproline (HURYCH and CHVAPIL 1962 b) and similar results were later obtained with intact chick embryos also (refer HURYCH and CHVAPIL 1962 b) The findings that the concentrations of free hydroxyproline in the serum (KOBRLE and CHVAPIL 1961 b) and in several organs (KOBRLE and CHVAPIL 1962) of rats decreased with the aging of the animals were also consistent with the above hypothesis Since the differences in the concentration of free hydroxyproline in different organs were better correlated with the metabolic activity of the organs than with their collagen contents it was further suggested that the concentration of free hydroxyproline in the tissues of animals of the same age is indirectly dependent on the metabolic activity of the collagen in the organ investigated (KOBRLE and CHVAPIL 1962)

A similar parallelism to that found between deoxyribonucleic acids (cells) and ultrafiltrable hydroxyproline in the carrageenin granuloma (CHVAPIL and ČMUCHALOVÁ 1960 1961) has also been found between deoxyribonucleic acids and free hydroxyproline during the development of polyvinyl sponge implant connective tissue by WOESSNER and BOUCEK (1961) They proposed that there might normally be a constant amount of free hydroxyproline within the fibroblasts In the experiments of DAUGHADAY and MARIZ (1962 b) in which cartilage was incubated *in vitro* with ^{14}C proline the specific activity of free hydroxyproline was 20 times as high as that of neutral salt soluble collagen hydroxyproline suggesting the origin of free hydroxyproline from activated hydroxyproline in this system In the intact chick embryo however the specific activity of free hydroxyproline was considerably lower than that of microsomal or soluble collagen hydroxyproline indicating that in the chick embryo free hydroxyproline may be formed by degradation of some of the more highly labelled protein fractions (PROCKOP PETERKOFSKY and UDENFRIEND 1962)

In relation to the problem of the significance of free and peptide bound hydroxyproline some investigations concerning the urinary hydroxyproline are also of interest ZIFF KIBRICK DRESNER and GRIBETZ (1956) who found that the excretion of urinary hydroxyproline decreases with age suggested that the urinary hydroxyproline may be derived from metabolically active forms of collagen such as the soluble collagens It has been suggested that the free hydroxyproline of urine may be derived from

building blocks of collagen and peptide bound hydroxyproline from the catabolism of collagen fibres (GERBER GERBER and ALTMAN 1960 GERBER and ALTMAN 1961) But these results could not be confirmed by LINDSTEDT and PROCKOP (1961) who found that the specific activities of free and peptide bound hydroxyproline in the urine were the same after the administration of ¹⁴C proline They further showed that in young rats there are at least three pools of hydroxyproline in the urine with half lives of approximately one day 5 days and 50 to 100 days These half live suggested that the first two pools had their origin in various soluble collagens and the third pool in the insoluble collagen In the later work of PROCKOP (1962 b) comparisons were made between the specific activities of urinary hydroxyproline and with various collagen fractions of the skin In investigations dealing with the changes in the excretion of hydroxyproline in scurvy and lathyrism and in relation to growth (MARTIN MERGENHAGEN and PROCKOP 1961 JASIN and ZIFF 1962 JASIN FINK SMILEY and ZIFF 1962 JASIN FINK WISE and ZIFF 1962) it was also emphasized that urinary hydroxyproline reflects changes in body collagen and especially in the more active forms of collagen

Thus far the role of the various hydroxyproline containing fractions has been considered in relation to the synthesis and normal metabolism of collagen Finally the role of these fractions during increased catabolism will be discussed JACKSON (1957) proposed that the catabolism of collagen during the resorption of the carrageenin granuloma is initiated by conversion of fibrous collagen into soluble form since the contents of neutral salt soluble and citrate soluble collagen fractions were increased during the resorption of the granuloma This was confirmed by WOESSNER (1962) in the involuting uterus where the amount of soluble collagen per uterus increased An even greater increase occurred in the free hydroxyproline whereas the amount of peptide bound hydroxyproline was not significantly altered WOESSNER (1962) suggested that the resorption of uterine collagen is initiated by solubilisation of the collagen which thereafter undergoes thermal denaturation Acid cathepsins split the denaturated collagen into peptides and peptidases split the peptides into free amino acids

EFFECT OF CORTISONE AND HYDROCORTISONE ON THE METABOLISM OF COLLAGEN

Cortisone and hydrocortisone at least when administered in large doses have a general inhibitory effect on the metabolism of connective tissue This is evident from the decrease in the number of mast cells and the changes in their morphology and histochemistry the delay in the formation of granulation tissue and in the healing of wounds associated with impairment of their tensile strength and the inhibition of the metabolism of the mucopolysaccharides of the ground substance (for general reference see IVERSEN 1954 DORFMAN and SCHILLER 1958 ASBOE HANSEN 1958 1959) The investigations concerning the effect of cortisone and hydrocortisone on the content of fibroblasts in vitro are not quite consistent for some indicate a decrease but others no effect Nevertheless in healing wounds the content of fibroblasts is decreased when cortisone is given in doses sufficient to delay wound healing (for references see ASBOE HANSEN 1958 1959) In agreement with this the increase in the total amount of collagen is inhibited by cortisone and hydro-

cortisone during the formation of new connective tissue in the polyvinyl sponge implants (PERNOKAS EDWARDS and DUNPHY 1957) in the carrageenin granuloma (ROBERTSON and SANBORN 1958 FISHER and PAAR 1960) in healing skin wounds (SAKATA 1960 b) and during the early stages of fibrosis of the liver (WATERMAN 1951)

Conflicting results have been obtained in investigations into the effect of cortisone on the total amount of collagen in the more mature connective tissue SILBER and PORTER (1953) found that large doses of cortisone primarily led to extensive losses of nitrogen from the rat carcass resulting in a decrease in body weight Collagen seemed to be relatively stable but a relative and absolute increase was found in the collagen of the carcass Other investigations have similarly shown an increase in the amount of collagen in the skeletal muscle of mice (FRIBERG 1957) and in the stomach liver and bone of rats (KOWALEWSKI 1961) The investigations of SOBEL and MARMORSTON (1954) SOBEL GABAY and JOHNSON (1958) and SOBEL GABAY JOHNSON and HASSAN (1959) indicated that the collagen of the tissues of rats was relatively stable compared with the decrease in the content of hexosamine and thus that a decrease occurred in the ratio of hexosamine to collagen after administration of cortisone Nevertheless in the investigations of SAKATA (1960 a) SETHI RAMEY and HOUCK (1961) SMITH (1962 a & b) and HOUCK (1962) a decrease in the collagen content of the skin was observed after administration of cortisone or hydrocortisone

In 1951 ROBERTS KARNOFSKY and FRANKEL found a great increase in the content of free hydroxyproline in the tissues of chick embryo six to ten days after a single injection of cortisone acetate onto the chorioallantoic membrane The contrary was found however when hydrocortisone was given to pregnant rats for the concentrations of free and total hydroxyproline in the tissues of new born rats decreased but the concentration of free proline increased (CHVAPIL 1958) Therefore CHVAPIL (1959) repeated the experiments of ROBERTS KARNOFSKY and FRANKEL (1951) but this investigation likewise showed that six to ten days after the administration of cortisone acetate the concentrations of free and total hydroxyproline in the chick embryo were greatly increased The discrepancy could be explained however by taking into consideration the fact that the dose of cortisone acetate (1 mg) used in the experiments with chick embryos greatly reduced the weight of the treated embryos When the absolute amounts of free and total hydroxyproline per embryo were calculated a decrease was observed that tallied with the results obtained in rat embryos On the basis of these experiments CHVAPIL (1959) proposed that cortisone inhibits hydroxyproline synthesis possibly during the hydroxylation of proline

DAUGHADAY and MARIZ (1962 a & b) who incubated rat cartilage *in vitro* with ^{14}C proline in the presence of hydrocortisone did not find any inhibition in the amount or labelling of free hydroxyproline in the medium although the incorporation of ^{14}C proline into cartilage bound hydroxyproline was significantly inhibited They concluded that in this system cortisone inhibited the terminal steps of collagen synthesis and not the formation of the supposed hydroxyproline intermediate

In studies on urinary hydroxyproline therapeutic doses of cortisone have been found to have no effect on the excretion of hydroxyproline in patients with rheumatoid arthritis or normal subjects (ZIFF KIBRICK DRESNER and GRIBETZ 1956) Even large doses of cortisone did not change the concentration of free hydroxyproline in the blood or its excretion in the urine in 1 and 11 month old rats although the weight of the animals and the concentration of free proline in the blood decreased considerably (KIVIRIKKO and LIESMAA 1958)

During recent years attention has been paid to the effect of cortisone on the soluble collagen fraction SILKO SIVELA and KULONEN (1959) found that a decrease occurred in the content of alkali soluble collagen in the skin of guinea pigs after administration of hydrocortisone A similar decrease was later observed in the neutral salt soluble collagen fractions of rat skin following administration of hydrocortisone (SETHI RAMEY and HOUCK 1961) or cortisone acetate (SAKATA 1960 a GUNTHER and CARSTEN 1961) The results concerning the effect of hydrocortisone or cortisone on the citrate soluble collagen fraction were not so consistent for the two first mentioned investigations indicated an increase and the two last mentioned and the investigation of SMITH (1962 a) a decrease in this fraction The investigations of SETHI RAMEY and HOUCK (1961) and of SMITH (1962 b) further indicated that the effects of hydrocortisone and cortisone on the cutaneous collagen are dependent on the age of the animals although as regards the nature of this age effect the results were not entirely consistent

In the study of GUNTHER and CARSTEN (1961) the effects of adrenalectomy and aldosterone were also investigated The contents of the various soluble collagen fractions increased after adrenalectomy while administration of aldosterone had no effect Since the increase after adrenalectomy was not greater in the 0 14 M sodium chloride soluble collagen fraction than in the other fractions it was argued that this effect was not due to increased synthesis of collag n but possibly to altered stability of the collagen due to changes in the composition or degree of polymerization of the mucopolysaccharides of the ground substance

MAZUROV and OREHOVIČ (1960) have studied the effect of cortisone on the incorporation of C glycine into the citrate soluble procollagen fraction of the skin The results indicated that cortisone inhibited the synthesis of procollagen and under certain conditions the incorporation of [14] C glycine was even reduced by half In the investigations of DAUGHADAY and MARIZ (1962 a & b) to which reference has already been made it was also evident that hydrocortisone inhibited the incorporation of [14] C proline into cartilage bound hydroxyproline in vitro By contrast the investigations of KOBLET and FRIEDEN (1960) revealed no inhibition of the incorporation of C glycine into the protein fractions of polyvinyl sponge implant connective tissue

EFFECT OF OTHER FACTORS ON THE METABOLISM OF COLLAGEN

In this section a brief survey will be given of the effects of other factors known to influence the metabolism of collagen The following factors are included in the present review age growth hormones a orbic acid and experimental lathyrism The effect of most of these factors on the metabolism of connective tissue have been recently reviewed by SMILEY and ZIFF (1962)

Age growth and growth ho mon — The contents of soluble collagens in the tissues (NAGEOTTE and GUYON 1934 K D OREHOVIČ 1950 BANFIELD 1952 KOBRLE and CHVAPIL 1958 BOUCEK NOBLE KAO and ELDEN 1958 KAO and MCGAVACK 1959 KOHN and ROLLERSON 1959 BAKERMAN 1962) the contents of free hydroxyproline in the blood (KOBRLE and CHVAPIL 1961 b) and in the tissues (CADAVID DENDUCHIS and MANCINI 1961 KOBRLE and CHVAPIL 1962) and the excretion of hydroxyproline in the urine (ZIFF KIBRICK DRESNER and GRIBETZ 1956 KIVIRIKKO and LIESMAA

cortisone during the formation of new connective tissue in the polyvinyl sponge implants (PERNOKAS, EDWARDS and DUNPHY 1957) in the carrageenin granuloma (ROBERTSON and SANBORN 1958, FISHER and PAAR 1960) in healing skin wounds (SAKATA 1960 b) and during the early stages of fibrosis of the liver (ATERMAN 1954)

Conflicting results have been obtained in investigations into the effect of cortisone on the total amount of collagen in the more mature connective tissue. SILBER and PORTER (1953) found that large doses of cortisone primarily led to extensive losses of nitrogen from the rat carcass resulting in a decrease in body weight. Collagen seemed to be relatively stable but a relative and absolute increase was found in the collagen of the carcass. Other investigations have similarly shown an increase in the amount of collagen in the skeletal muscle of mice (FRIBERG 1957) and in the stomach, liver and bone of rats (KOWALEWSKI 1961). The investigations of SOBEL and MARMORSTON (1954), SOBEL, GABAY and JOHNSON (1958) and SOBEL, GABAY, JOHNSON and HASSAN (1959) indicated that the collagen of the tissues of rats was relatively stable compared with the decrease in the content of hexosamine and thus that a decrease occurred in the ratio of hexosamine to collagen after administration of cortisone. Nevertheless, in the investigations of SAKATA (1960 a), SETHI, RAMEY and HOUCK (1961), SMITH (1962 a & b) and HOUCK (1962) a decrease in the collagen content of the skin was observed after administration of cortisone or hydrocortisone.

In 1951 ROBERTS, KARNOFSKY and FRANKEL found a great increase in the content of free hydroxyproline in the tissues of chick embryo six to ten days after a single injection of cortisone acetate onto the chorioallantoic membrane. The contrary was found, however, when hydrocortisone was given to pregnant rats, for the concentrations of free and total hydroxyproline in the tissues of new born rats decreased but the concentration of free proline increased (CHVAPIL 1958). Therefore CHVAPIL (1959) repeated the experiments of ROBERTS, KARNOFSKY and FRANKEL (1951) but this investigation likewise showed that six to ten days after the administration of cortisone acetate the concentrations of free and total hydroxyproline in the chick embryo were greatly increased. The discrepancy could be explained, however, by taking into consideration the fact that the dose of cortisone acetate (1 mg) used in the experiments with chick embryos greatly reduced the weight of the treated embryos. When the absolute amounts of free and total hydroxyproline per embryo were calculated, a decrease was observed that tallied with the results obtained in rat embryos. On the basis of these experiments CHVAPIL (1959) proposed that cortisone inhibits hydroxyproline synthesis, possibly during the hydroxylation of proline.

DAUGHADAY and MARIZ (1962 a & b) who incubated rat cartilage *in vitro* with ^{14}C proline in the presence of hydrocortisone did not find any inhibition in the amount or labelling of free hydroxyproline in the medium, although the incorporation of ^{14}C proline into cartilage *bound hydroxyproline was significantly inhibited*. They concluded that in this system cortisone inhibited the terminal steps of collagen synthesis and not the formation of the supposed hydroxyproline intermediate.

In studies *on urinary hydroxyproline*, therapeutic doses of cortisone have been found to have no effect on the excretion of hydroxyproline in patients with rheumatoid arthritis or normal subjects (ZIFF, KIBRICK, DRESNER and GRIBETZ 1956). *Even large doses of cortisone did not change the concentration of free hydroxyproline in the blood or its excretion in the urine in 1 and 11 month old rats, although the weight of the animals and the concentration of free proline in the blood decreased considerably* (KIVIRIKKO and LIESMAA 1958).

Parathyroid hormone — The excretion of hydroxyproline in the urine (KLEIN ALBERTSEN and CURTISS 1962 DULL CALSING and HENNEMAN 1962) and the con centration of hydroxyproline in the plasma (BATES McGOVEN and TALMAGE 1962) are increased in hyperparathyroidism The incorporation of ¹⁴C proline into ¹⁴C hydroxyproline *in vitro* has been found to be reduced in the bones of rats treated with parathyroid extract although the incorporation of ¹⁴C 2 glucose into the hexosamine of the bone matrix was increased in similarly treated animals (JOHNSTON DEISS and MINER 1962)

Steroid hormones — The effect of cortisone and hydrocortisone on the metabolism of collagen has already been described (pp 13—15) As regards the other anti inflam matory steroids it has been found that 6 methyl prednisolone inhibited collagen formation in polyvinyl sponge implant connective tissue and that growth hormone methyl testosterone or stilboestrol were not able to counteract this inhibition (BAVETTA BEKHOR and NIMNI 1962) KUHN HOLZMANN and KORTING (1962) have reported that the contents of 0 5 M sodium chloride soluble and citrate soluble col lagen were decreased in the skin of rats by administration of prednisolone

The effect of deoxycorticosterone acetate has been reported to be the opposite of that of cortisone in carrageenin granulomas for it increased collagen formation (ROBERTSON and SANBORN 1958) and the weight (ATKINSON JENKINS TOMICH and WOOLLETT 1962) of the granulomas In the skin of rats however the effect of deoxycorticosteroid was similar to that of cortisol with the exception of aged animals both hormones causing a decrease in the contents of neutral salt soluble and insoluble collagen and an increase in the content of citrate soluble collagen (SETHI RAMEY and HOUCK 1961)

Anabolic steroids increased the tensile strength of healing fracture callus of bone in normal rats (WIANCO and KOWALEWSKI 1961) and the tensile strength and collagen content of experimental granulation tissue in undernourished rats in which forma tion of connective tissue was retarded (VILJANTO ISOMAKI and KULONEN 1962)

Collagen formation in polyvinyl sponge implant connective tissue is dependent on the sex of the host males displaying a more active synthesis of collagen and forma tion of larger amounts of stable collagen than females (KAO BOUCEK and NOBLE 1957 BOUCEK NOBLE and WOESSNER 1959 a & b BOUCEK and NOBLE 1961) Accord ing to BAVETTA BEKHOR and NIMNI (1962) stilboestrol inhibits collagen formation in the polyvinyl sponge implant connective tissue whereas ROBERTSON and SANBORN (1958) have found enhanced collagen formation in the carrag nin granuloma after the administration of stilboestrol KUHN HOLZMANN and KORTING (1962) have reported that the administration of testosterone increased the content of 0 5 M sodium chloride solubl collag n in the skin

Asco bic acid — Several investigations have shown that collagen formation is reduced in ascorbic acid deficiency The mechanism of action of ascorbic acid on collagen formation is still unknown however (for general references see GOULD 1960 ROBERTSON 1961) In s orbutic guinea pigs the contents of soluble collagens in the skin (OREHOVIĆ and SPIKITER 1957 GROSS 1959 b SAKATA 1960 a) and wound tissue (SAKATA 1960 b) the contents of free and peptide bound hydroxyproline in granulomas (ČMUCHALOVA and CHVAPIL refer KOBRLE and CHVAPIL 1962) and the excretion of hydroxyproline in the urine (MARTIN MERGENHAGEN and PROCKOP 1961) are decreased It has been suggested that ascorbic acid is needed for hydroxyla ti n of peptide bound proline (ROBERTSON and SCHWARTZ 1953 GOULD and WOESS

1958, Lindstedt and Prockop 1961, Martin, Mergenhagen and Prockop 1961) decrease during the aging of animals and human subjects. The content of 0.45 M sodium chloride soluble collagen in the skin of guinea pigs, however, is dependent not only on the age but also on the growth rate (Gross 1958 b), and the same has also been observed concerning the excretion of hydroxyproline in the urine (Jasin, Fink, Smiley and Ziff 1962, Jasin, Fink, Wise and Ziff 1962). In agreement with this Wirtschafter and Bentley (1962 a) have found that the maximum content of 0.45 M sodium chloride soluble collagen in the skin of rats occurred at the age of 35 days, when the rate of weight gain was likewise greatest. Administration of growth hormone to experimental animals is followed by an increase in the content of acid soluble collagen in the skin (Banfield 1958) and in the content of free hydroxyproline in the blood (Kivirikko, Liesmaa and Luukkainen 1958). Furthermore, growth hormone administered to patients with hypopituitarism increases the excretion of hydroxyproline in the urine (Jasin, Fink, Smiley and Ziff 1962, Jasin, Fink, Wise and Ziff 1962).

The above mentioned findings have several times been taken to indicate a decrease in collagen metabolism with age or with cessation of growth and an increase in collagen metabolism after administration of growth hormone. This is also evident from the isotope experiments of Neuberger, Perrone and Slack (1951), Neuberger and Slack (1953) and Kao, Hilker and McGavack (1961), which indicated that the rate of turnover of collagen is greater in young than in old animals. The isotope experiments of Lindstedt and Prockop (1961) further showed that there are larger hydroxyproline pools with short half lives in young than in old animals. In experiments in vitro Daughaday and Mariz (1962 a) have found that the rate of formation of collagen is reduced in the cartilage of hypophysectomized animals and increased in that of animals treated with growth hormone.

Thyroid hormones and thyroid stimulating hormone. — Thyroid hormones have an inhibitory effect on the formation of collagen as measured by the tensile strength of healing wounds (Moltke 1958). In the aortic walls of normal rabbits (Lorenzen 1961) and in the bones of normal rats (Gabay, Vivanco, Ramos and Diaz 1961) no change in the total collagen content has been observed after administration of thyroxine, although in the latter investigation thyroxine inhibited the increase in the collagen caused by methylene aminoacetonitrile. The excretion of hydroxyproline in the urine is increased in normal human subjects and young and old rats after the administration of thyroid hormones (Keiser and Sjoerdsma 1962, Kivirikko, Koivusalo, Laitinen and Liesmaa 1963) and in patients with hyperthyroidism (Dull and Henneman 1963, Kivirikko, Koivusalo, Laitinen and Lamberg, to be published) and reduced in adult patients with hypothyroidism (Keiser and Sjoerdsma 1962) and in children with cretinism (Jasin, Fink, Smiley and Ziff 1962, Jasin, Fink, Wise and Ziff 1962). The contents of 0.45 M sodium chloride soluble and citrate soluble collagen in the skin of young rats have been found to be slightly decreased after the administration of thyroxine (Kivirikko, Koivusalo, Laitinen and Liesmaa 1963).

Besides its stimulatory effect on the thyroid gland, thyroid stimulating hormone has a direct stimulatory effect on the metabolism of the mucopolysaccharides of the ground substance (see Asboe-Hansen 1958, Smiley and Ziff 1962), but the possible direct effect of this hormone on the metabolism of collagen is unknown (Smiley and Ziff 1962).

genic agents act by blocking carbonyl groups on the collagen molecule and thus preventing the cross linking essential to normal maturation Further support for this hypothesis was obtained in the same investigation from the finding that purified lathyrit c collagen took up mall r amounts of 2 4 dinitrophenylhydrazine than normal collagen In addition STALDER and STEGEMANN (1963) have reported that β aminopropionitrile is built up into the collagen molecule for β alanine which is formed during the hydrolysis of β aminopropionitrile was found in the hydrolysates of acid soluble collagen obtained from lathyritic animals but not in those obtained from normal animals

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According to NEUMAN (1950) hydroxyproline was not found in crude hen s egg contents except in the egg shell membranes where its content was 0 66—1 0 per cent Since the content of hydroxyproline in these membranes did not change during embryonic development it was concluded that the hydroxyproline of the chick embryo cannot be derived in appreciable amounts from the egg contents Recently FUJIMOTO and TAMIYA (1962) have reported a value of 270 µg/egg for hydroxyproline in the crude egg contents but even this value is too low to be a noteworthy source of hydroxyproline for the embryo

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salt soluble and citrate soluble collagen were present and were able to form fibrils. They also contained more hydroxyproline than corresponding fractions from embryos 6—8 cm long. Since the neutral salt soluble collagen of the smaller embryos could not form fibrils it was suggested that new collagen fibrils are formed by envelopment of precollagenous filaments with soluble collagen molecules rather than by coprecipitation. It was further pointed out that mucoproteins must play a noteworthy role in this mechanism.

embryo may be derived from some of the more highly labelled collagen fractions Neither was there any incorporation of free hydroxyproline into the microsomal protein of the cell free system obtained from chick embryo microsomes (PETERKOFSKY and UDENFRIEND 1961)

There are only a few reports in the literature concerning the content of peptides containing hydroxyproline in the chick embryo DECKER LEVENE and GROSS (1959) have found that the content of acid extractable peptides was very low in 10 and 17 day old chick embryos The values for ultrafiltrable peptides in chick embryo lung fibroblast cultures were also considerably lower than the values for free hydroxyproline (CHVAPIL HOLEČKOVÁ ČMUCHALOVÁ KOBRLE and HURYCH 1962) When the skin of chick embryos was incubated *in vitro*, however the content of ultrafiltrable peptides was 30 to 60 per cent of the total ultrafiltrable hydroxyproline and the total ultrafiltrable fraction showed a high metabolic activity (HURYCH and CHVAPIL 1962 b)

The contents of soluble collagens have not been studied systematically during chick embryo development HERRMANN and BARRY (1955) first suggested that during some phases of development a form of collagen is present which can be extracted with 0 1 N sodium hydroxide although their attempts to extract citrate soluble collagen were inconclusive LEVENE and GROSS (1959) studying the effect of β aminopropionitrile on collagen in chick embryos reported values of 27 0 16 5 and 20 0 μg/ml for hydroxyproline in crude 1 M sodium chloride extracts (2 ml of 1 M sodium chloride per g of fresh tissue) of skin bone and aorta in 17 day old control embryos About 7 μg/ml of these values represented free hydroxyproline They further reported that collagen could not be extracted from normal skin or bone with acid citrate buffer or 0 5 per cent acetic acid Some other investigations on lathyrism have also given values for hydroxyproline in crude 1 M sodium chloride extract of control embryos at some stage of development (e g LEVENE 1961 a SMILEY YEAGER and ZIFF 1962) The values for nondialyzable 1 M sodium chloride soluble hydroxyproline reported by SMITH and SHUSTER (1962) in 15 day old embryos were considerably lower than those presented by other investigators for the crude 1 M sodium chloride soluble fraction

There are also some reports in the literature concerning the occurrence of hydroxyproline in other embryos In the frog embryo hydroxyproline is not present during the earliest stages of development and only appears at stage 21 or 22 (EDDS 1958) As in chick embryos the content of free hydroxyproline is relatively high in rat (CHVAPIL 1958) and pig (KOBRLE and CHVAPIL 1961 a) embryo In rats the content of 0 45 M sodium chloride soluble collagen is considerably lower in embryos than after birth (WIRTSCHAFTER and BENTLEY 1962 a) In human embryos the maximum content of citrate soluble hydroxyproline is reached several weeks before birth and a steady decrease then occurs with age (BAKERMAN 1962) Both free and bound hydroxyproline are present in human amniotic fluid and there is a significant decrease in the content of free hydroxyproline and in the ratio of free to bound hydroxyproline from early to full term pregnancy (KIVIRIKKO KOIVUSALO and KOIVUSALO 1963)

TUSTANOVSKIJ ZAJDES ORLOVSKAJA and MJACKAJA (1961) have studied the collagen components in the skin of pig embryos of two age groups In 6—8 cm long embryos the neutral salt soluble collagen was unable to form fibril and it had high proline hydroxyproline and glycine hydroxyproline ratios The content of citrate soluble collagen was negligible at this stage In embryos 19 cm long both neutral

— The specific activities of hydroxyproline in free hydroxyproline in hydroxyproline of the 1 M sodium chloride-soluble collagen and in the insoluble fraction after administration of ^{14}C proline

In the last part of the investigation the cortisone induced inhibition of collagen formation is studied The following effects were studied

— The effect on the hydroxyproline-containing fractions in whole embryos

— The effect on the hydroxyproline-containing fractions in the skin of the embryos

OUTLINES OF PRESENT INVESTIGATION

When the preliminary experiments for the present investigation were started in 1959 there were only a few reports in the literature concerning the low molecular hydroxyproline-containing fractions It was known however that tissues of chick embryos have a high content of free hydroxyproline Since it was also known that hydroxyproline is not present in appreciable amounts in the egg chick embryos seemed to be suitable as material for investigations dealing with the changes in the various hydroxyproline-containing fractions during the formation of collagen

The purpose of the present investigation was to make a simultaneous study of the low-molecular and macromolecular hydroxyproline-containing fractions during the formation of collagen in chick embryos The following fractions were studied free hydroxyproline peptide hydroxyproline 1 M sodium chloride soluble collagen hydroxyproline, citrate soluble collagen hydroxyproline insoluble hydroxyproline and total hydroxyproline The changes in the amounts of these fractions were considered not only in relation to dry weight but also as percentages of the total hydroxyproline

The purposes of the first part of the investigation were as follows

— to test the reliability of the methods for the determination of hydroxyproline in the fractions in question

— to work out a suitable procedure for the preparation of the various fractions from the same tissue sample

— to study the reliability of this fractionation method

The next part of the investigation deals with the properties of the various hydroxyproline-containing fractions during the formation of collagen in normal chick embryos The following points were studied

— The changes occurring in the hydroxyproline containing fractions in whole embryos during embryonic development

— The changes taking place in the hydroxyproline-containing fractions of the skin to discover whether the changes observed in whole embryos are similar to those occurring in tissues rich in collagen

(KIVIRIKKO and LIESMAA 1959) were intended to increase the stability and sensitivity of the oxidation and colour formation

In the present investigation two methods have been used for the determination of hydroxyproline These were the method suggested by KIVIRIKKO and LIESMAA (1959) with some further modifications and procedure II of the method suggested by PROCKOP and UDENFRIEND (1960) The latter method was used for the determination of hydroxyproline in the 1 M sodium chloride-soluble collagen hydroxyproline fraction and for the determination of hydroxyproline in all fractions of embryos up to and including the 8th day of development since in these cases the content of hydroxyproline compared with that of other substances was too low to be determined by the former method In other cases the former method was used since it was considerably more rapid and yet gave results identical with the latter A more detailed description of the methods is given in the following

The method of KIVIRIKKO and LIESMAA (1959) with some further modifications

The following slightly modified procedure was used in the present investigation

Reagents

Permutit — Permutit ammoniakfrei nach Folin (Th Schuchardt GMBH & Co München) or Permutit Folin (The Permutit Company Ltd London) were used

Hydroxyproline standards — Standards containing 1 µg/ml or 2 µg/ml were diluted daily from standard stock solution containing 1 mg/ml (stored at + 2 C)

Sodium hypobromite — 3 2 ml of bromine was added to 500 ml of ice cold 5 per cent sodium hydroxide This stock solution which has a bromine content of 2 per cent (wt /vol) was stored at + 2 C and only used after one week The solution was then stable for several months Always on the evening of the day before the analysis a portion of this solution was diluted with cold 5 per cent sodium hydroxide to a bromine content of 0 35 per cent and then also stored at + 2 C

Sodium sulphite — 3 per cent (wt /vol) in distilled water

p Dimethylaminobenzaldehyde — 5 per cent (wt /vol) in n propanol

Hydrochloric acid — 6 N

Procedure

Solutions containing 1—3 µg/ml of hydroxyproline were shaken with approximately one tenth their weight of permutit for 5 minutes After filtration the clear solutions were used for analysis

Into test tubes standing in ice water 2 ml of the unknown solution or standard containing 1 µg/ml or 2 µg/ml of hydroxyproline or distilled water for blank determination were pipetted Analyses were mostly carried out in series of 36 tubes Then

MATERIAL AND METHODS

ANALYTICAL METHODS

DETERMINATION OF HYDROXYPROLINE

Most colorimetric methods for the determination of hydroxyproline are based on the oxidation of hydroxyproline to pyrrole or pyrrole 2-carboxylic acid which is then condensed with p dimethylaminobenzaldehyde to give a coloured compound

The first methods were developed by LANG (1933) and by WALDSCHMIDT LEITZ and AKABORI (1934) In these methods sodium hypochlorite was used for oxidation and pyrrole was separated from the reaction mixture by distillation before colour formation The method of WISS (1949) which also involved oxidation with sodium hypochlorite and distillation of pyrrole before the colour reaction was used for the determination of hydroxyproline in blood and urine Further modifications of this method have been suggested by MITOMA SMITH DAVIDSON UDENFRIEND DACOSTA and SJOERDSMA (1959)

In the method of NEUMAN and LOGAN (1950) hydroxyproline is oxidized with hydrogen peroxide in the presence of copper sulphate and sodium hydroxide to Δ^1 pyrroline 4 carboxylic acid (see RADHAKRISHNAN and MEISTER 1957) which is then condensed with p dimethylaminobenzaldehyde in n propanol for colour formation The method has the disadvantages that an interfering colour is formed with tyrosine and tryptophan and that there is some variability in the colour reaction with hydroxyproline Therefore several modifications of this method have been suggested in order to improve the specificity and stability of the colour formation (e g MARTIN and AXELROD 1953 MIYADA and TAPPEL 1956 FELS 1958 MITOMA et al 1959 BAILY 1960 HUTTERER and SINGER 1960 LEACH 1960)

In the method of STEGEMANN (1958) chloramine T is used for oxidation of hydroxyproline to pyrrole Tyrosine and tryptophan do not give an interfering colour Further modifications of this method have been suggested by WOESSNER (1961)

PROCKOP and UDENFRIEND (1960) who also used chloramine T for oxidation have investigated the conditions of oxidation and colour formation with respect to great excesses of other amino acids Their method can be applied directly to the analysis of hydroxyproline in solutions containing less than 0 01 per cent hydroxyproline including urine

MUTING (1952) developed a method in which sodium hypobromite was used for the oxidation of hydroxyproline The modifications to this method suggested later

(Kivirikko and Liesmaa 1959) were intended to increase the stability and sensitivity of the oxidation and colour formation

In the present investigation two methods have been used for the determination of hydroxyproline These were the method suggested by Kivirikko and Liesmaa (1959) with some further modifications and procedure II of the method suggested by Prockop and Udenfriend (1960) The latter method was used for the determination of hydroxyproline in the 1 M sodium chloride soluble collagen hydroxyproline fraction and for the determination of hydroxyproline in all fractions of embryos up to and including the 8th day of development since in these cases the content of hydroxyproline compared with that of other substances was too low to be determined by the former method In other cases the former method was used since it was considerably more rapid and yet gave results identical with the latter A more detailed description of the methods is given in the following

The method of Kivirikko *and* Liesmaa *(1959) with some further modifications*

The following slightly modified procedure was used in the present investigation

Reagents

Permutit — Permutit ammoniakfrei (nach Folin (Th Schuchardt GMBH & Co Munchen) or Permutit Folin (The Permutit Company Ltd London) were used

Hydroxyproline standards — Standards containing 1 µg/ml or 2 µg ml were diluted daily from standard stock solution containing 1 mg/ml (stored at + 2 C)

Sodium hypobromite — 3 2 ml of bromine was added to 500 ml of ice cold 5 per cent sodium hydroxide This stock solution which has a bromine content of 2 per cent (wt /vol) was stored at + 2 C *and only used after one week* The solution was then stable for several months Always on the evening of the day before the analysis a portion of this solution was diluted with cold 5 per cent sodium hydroxide to a bromine content of 0 35 per cent and then also stored at + 2 C

Sodium sulphite — 3 per cent (wt /vol) in distilled water

p Dimethylaminobenzaldehyde — 5 per cent (wt /vol) in *n* propanol

Hydrochloric acid — 6 N

Procedure

Solutions containing 1—3 µg/ml of hydroxyproline were shaken with approximately one tenth their weight of permutit for 5 minutes After filtration the clear solutions were used for analysis

Into test tubes standing in ice water 2 ml of the unknown solution or standard containing 1 µg/ml or 2 µg/ml of hydroxyproline or distilled water for blank determination were pipetted Analyses were mostly carried out in series of 36 tubes Then

MATERIAL AND METHODS

ANALYTICAL METHODS

DETERMINATION OF HYDROXYPROLINE

Most colorimetric methods for the determination of hydroxyproline are based on the oxidation of hydroxyproline to pyrrole or pyrrole 2 carboxylic acid which is then condensed with *p* dimethylaminobenzaldehyde to give a coloured compound

The first methods were developed by LANG (1933) and by WALDSCHMIDT LEITZ and AKABORI (1934) *In these methods sodium hypochlorite was used for oxidation* and pyrrole was separated from the reaction mixture by distillation before colour formation The method of WISS (1949) which also involved oxidation with sodium hypochlorite and distillation of pyrrole before the colour reaction was used for the determination of hydroxyproline in blood and urine Further modifications of this method have been suggested by MITOMA SMITH DAVIDSON UDENFRIEND DACOSTA and SJOERDSMA (1959)

In the method of NEUMAN and LOGAN (1950) hydroxyproline is oxidized with hydrogen peroxide in the presence of copper sulphate and sodium hydroxide to Δ^1 pyrroline 4 carboxylic acid (see RADHAKRISHNAN and MEISTER 1957) which is then condensed with *p* dimethylaminobenzaldehyde in *n* propanol for colour formation The method has the disadvantages that an interfering colour is formed with tyrosine and tryptophan and that there is some variability in the colour reaction with hydroxyproline Therefore several modifications of this *method have been* suggested in order to improve the specificity and stability of the colour formation (*e g* MARTIN and AXELROD 1953 MIYADA and TAPPEL 1956 FELS 1958 MITOMA *et al* 1959 BAILY 1960 HUTTERER and SINGER 1960 LEACH 1960)

In the method of STEGEMANN *(1958) chloramine* T *is used for oxidation* of hydroxyproline to pyrrole Tyrosine and tryptophan do not give an interfering colour Further modifications of this method have been suggested by WOESSNER (1961)

PROCKOP and UDENFRIEND (1960) who also used chloramine T for oxidation have investigated the conditions of oxidation and colour formation with respect to great excesses of other amino acids Their method can be applied directly to the analysis of hydroxyproline in solutions containing less than 0 01 per cent hydroxyproline including urine

MÜTING (1952) developed a method *in which sodium hypobromite was used for* the oxidation of hydroxyproline The modifications to this method suggested later

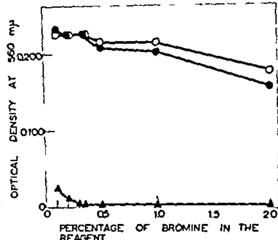


Fig. 1 Effect of bromine content in the sodium hypobromite reagent on colour formation with 4 μg of hydroxyproline in distilled water ○ with 4 μg of hydroxyproline added to 1000 μg of casein amino acids ● and with 1000 μg of casein amino acids ▲

Accuracy and specificity

If the method was giving optimal values a standard containing 4 μg of hydroxyproline (ml of standard containing 2 μg/ml) gave an optical density of 0 220 Somewhat lower optical densities were often obtained especially when the hypobromite stock standard was newly prepared It was found however that the recoveries of hydroxyproline added to protein hydrolysates were 100 per cent provided the standard gave an optical density of more than 0 190 The standard deviation calculated from the 200 last duplicate analyses representing the various fractions was ± 3 1 per cent If the deviation between the duplicates of one sample exceeded 10 per cent the sample was reanalysed

The sodium chloride formed during neutralization of the samples did not have an effect on the recoveries since even 170 mg of sodium chloride added to 4 μg of hydroxyproline permitted a recovery of 100 per cent The recoveries were tested several times during the present investigation by adding varying amounts of hydroxyproline to the solutions to be analysed and invariably a recovery of 95 to 103 per cent was obtained

The specificity of the method was tested with other amino acids including tyrosine and tryptophan but no appreciable interfering colour was observed The absorption spectra were often determined for the coloured compound obtained with the present method in various fractions but peaks that might have given a significant nonspecific colour at 560 mμ were not generally observed Only in the 1 M sodium chloride soluble collagen hydroxyproline fraction was a peak observed at 460 mμ which could possibly have given about 10 per cent unspecific colour at 560 mμ Therefore after the preliminary experimental series this method was not used for the determination of 1 M sodium chloride soluble collagen hydroxyproline

Final evidence of the reliability of the method was obtained by analysing the same samples by the method of PROCKOP and UDENFRIEND (1960) Identical values were obtained in all fractions except the 1 M sodium chloride soluble collagen hydroxyproline fraction where the method of PROCKOP and UDENFRIEND (1960) gave lower values than the present method This was also true of the other fractions in embryos up to the 8th day of development

tube no 1 contained the blank determination tubes nos 2 and 25 standard 1 μg/ml and tubes nos 11 18 and 35 standard 2 μg/ml The samples were pipetted into the other tubes in duplicate in such a way that the duplicate values were not in adjacent tubes When the solutions were definitely ice cold 2 ml of sodium hypobromite was added with an ice cold pipette to twelve test tubes The pipette was emptied without blowing so as to prevent its warming and the tubes were then shaken The pipette was re cooled by drawing up cold hypobromite several times and allowing it to run out again and hypobromite was then added to the following twelve test tubes When these tubes had been shaken the last twelve tubes received their hypobromite About 3 to 10 min after the addition of the hypobromite 0 1 ml of sodium sulphite was added This was followed by 2 ml of p dimethylaminobenzaldehyde reagent and by 1 ml of 6 N HCl The tubes were shaken immediately after each addition The tubes were then transferred from ice water to a boiling water bath for 2 min 30 sec During this time cold air was blown onto the upper parts of the tubes to prevent evaporation After cooling in cold water for 3 min the tubes were allowed to stand at room temperature for 15—60 min before the optical densities were read at 560 mμ in a Beckman model B spectrophotometer in 10 mm cuvettes

Optimum conditions for the method

When our original method (KIVIRIKKO and LIESMAA 1959) was used it was found that especially with the newly prepared sodium hypobromite reagent recoveries slightly lower than 100 per cent were often obtained when hydroxyproline was added to protein hydrolysates This suggested further investigation of the oxidation reaction The values presented by PROCKOP and UDENFRIEND (1960) also indicated that the concentration of the oxidant is important for optimum recoveries

The optimum concentration of sodium hypobromite was investigated in experiments in which 2 ml of sodium hypobromite reagents of varying concentrations were added to 2 ml samples containing 4 μg of hydroxyproline in distilled water with or without addition of 1000 μg of acid casein hydrolysate (Casamino Acids Difco Laboratories Inc Detroit) or only 1000 μg of acid casein hydrolysate without hydroxyproline The optimum concentration of sodium sulphite needed for the destruction of hypobromite was first determined for each hypobromite concentration and in these experiments sodium sulphite was then added in the amounts optimal for each hypobromite concentration The sodium hypobromite reagent in our original method was prepared as suggested by MUTING (1952) by addition of 1 8 ml bromine to 500 ml of 5 per cent sodium hydroxide and accordingly had a bromine content of 1 125 per cent The results (Fig 1) indicated that the optimum concentration of sodium hypobromite was less than 1 125 per cent On the other hand if the concentration was too low casein amino acid hydrolysate in which no hydroxyproline is present began to give some colour In the light of these results a hypobromite reagent containing 0 35 per cent bromine was chosen for oxidation Since a more concentrated reagent is more stable when stored for any length of time a stock solution containing 2 per cent bromine in 5 per cent sodium hydroxide was used

The optimum amount of sodium sulphite was found to be 3 mg at this hypobromite concentration The amounts of other reagents were found to be optimal in our original method

obtained by the present fractionation method for the former values were only about 80 per cent of the latter in embryos 13 days of age Therefore it is not quite correct to compare the extents of the changes in the free hydroxyproline with those in the free proline or free amino nitrogen

Total nitrogen was determined by the micro Kjeldahl technique from an aliquot of the same neutralized and filtered sample as was used for the determination of total hydroxyproline and total proline This had the advantage that any errors in the pipetting of the homogenate would be reflected as similar changes in the total nitrogen Since the sample was taken after neutralization and filtration the unfiltrable part of the humin nitrogen was not included in the value

The relative dry weight of the embryo or of the skin was determined in a sample of the homogenate (p 30) transferred to a small tared beaker The beaker with its contents was weighed and then dried at 108 C for 24 hours The beaker was then weighed again and the wet and dry weights of the homogenate sample were calculated by subtracting the weight of the beaker The relative dry weight of the embryo or of the skin was then calculated from the following equation

$$D = \frac{b \quad 3\,08}{a} - 117$$

where D = relative dry weight in mg/g wet weight
a = wet weight of the homogenate sample (g)
b = dry weight of the homogenate sample (mg)
3 08 = the weight of the homogenate (g) containing 1 g of the tissue (specific gravity of 1 M NaCl = 1 04 1 g + 2 08 g = 3 08 g)
11 = dry weight of 2 ml of 1 M NaCl (mg)

Statistical methods The results are given in the tables as arithmetic means ± standard deviations

In that part of the investigation in which the effect of cortisone was studied the significance of the differences between the cortisone treated groups and the controls was estimated by Student s *t* test on the assumption that the variations of the cortisone treated and control groups are basically equal (see *e g* BANCROFT 1957 p 176)

MATERIAL

Fertilized eggs of white Leghorn chickens weighing 55 ± 2 grams were purchased from Saarioinen Oy Sahalahti They were placed in an incubator of 120 egg capacity at a temperature of 37 5 ± 0 5 C and relative humidity of 65 ± 5 per cent in which the air was kept moving with the aid of an electric fan The eggs were supported in trays containing 30 eggs with the air chamber upwards the axis of the egg forming an angle of 30 with the vertical and they were turned once daily At the 8th or 9th day of incubation the eggs were tested for fertility with a lamp and infertile eggs were removed

Injections of substances were made through a small hole in the egg shell into the air chamber (onto the chorioallantoic membrane) after which the hole was covered with adhesive tape and incubation continued In the experiments with cortisone the eggs were so arranged in the trays that the control eggs were not adjacent to each other but among the cortisone treated ones

The method of PROCKOP *and* UDENFRIEND *(1960)*

In procedure II of this method hydroxyproline is oxidized to Δ^1 pyrroline 4 hydroxy 2 carboxylic acid and pyrrole 2 carboxylic acid with chloramine T in the presence of great excess of alanine Since these first oxidation products are not soluble in toluene substances that might interfere with colour formation can be removed by extraction with toluene The tubes are then placed in a boiling water bath to form pyrrole from the first oxidation products Since pyrrole is soluble in toluene it can be extracted with toluene from the reaction mixture Colour is finally formed by addition of *p* dimethylaminobenzaldehyde reagent in acid ethanol to the toluene containing pyrrole

The only modification made to this method was that all volumes were reduced to one half Besides increasing sensitivity this had the advantage that the procedure could now be carried out in screw capped tubes of the same size as ordinary test tubes Thus it was easy to work with a series of 36 test tubes in the same rack

In the case of the samples that were neutralized to pH 4—6 with universal indicator paper (p 33) the sample taken for analysis was further neutralized with 0 05 N potassium hydroxide to a faint pink colour of phenolphthalein as described in the method

DETERMINATION OF THE SPECIFIC ACTIVITY OF HYDROXYPROLINE

For the determination of the specific activity of hydroxyproline the method of PROCKOP UDENFRIEND and LINDSTEDT (1961) was used In this method as in the method of PROCKOP and UDENFRIEND (1960) hydroxyproline is first oxidized to *oxidation products that are insoluble in toluene Interfering substances are removed* by repeated extraction with chloroform and toluene and the first oxidation products are then converted to pyrrole which is extracted with toluene An aliquot of the *toluene extract is taken for pyrrole assay* and another aliquot for counting in a liquid scintillation counter with 2 5 diphenyloxazole and *p* bis(2 (5 phenyloxazolyl)) benzene as phosphor

In the present investigation an Ecko type 664 A liquid scintillation counter (Ecko *Electronics Ltd) was used The samples were counted to have an error of less than* 1 per cent The results are expressed as counts/min/μmole

OTHER METHODS

Proline was determined by the method of TROLL and LINDSLEY (1955)

Free amino nitrogen was determined by the ninhydrin method of RUBINSTEIN and PRYCE (1959)

Since the methods used *for the determination of free proline and free amino nitro* gen also give some reaction with peptides containing proline or amino nitrogen the values obtained do not represent simply free proline or free amino acids Because *the 80 per cent ethanol used in the fractionation procedure* of the present investiga tion extracted considerable amounts of peptides the error may be fairly considerable This was also evident from the experiments in which values obtained by direct *trichloroacetic acid precipitation* of the homogenate were compared with the values

was used for the determination of the 1 M sodium chloride soluble ethanol-insoluble hydroxyproline termed in the present investigation 1 M sodium chloride soluble collagen hydroxyproline

LEVENE and GROSS (1959) used 1 M sodium chloride solution buffered with phosphate buffer pH 7 6 ionic strength 0 02 Since it seemed probable that the pH values of the homogenates obtained from various chick embryos would not differ significantly from each other 1 M sodium chloride was used unbuffered in the present investigation in the same way as GROSS (1958 a) had earlier used 0 45 M sodium chloride solution Measurements of the pH of the 1 M sodium chloride soluble fraction made during the present study always gave values between 7 0 and 7 3 even if the homogenate was stored at + 2 C for 2 days indicating that buffering of the 1 M sodium chloride solution was unnecessary

In addition to the above mentioned fractions the value for total hydroxyproline was determined from the crude homogenate and after deduction of the values for other fractions the value for insoluble hydroxyproline was obtained by difference In those series in which peptide hydroxyproline was not determined the insoluble hydroxyproline was calculated as if no peptides had been present Since the values for peptide hydroxyproline were very small this method of calculation introduces an error of less than 1 per cent In the samples of 2¹ 3- and 4 day-old embryos the insoluble hydroxyproline was determined and total hydroxyproline was calculated

The citrate soluble collagen hydroxyproline (insoluble in 1 M sodium chloride) was only determined in a few series Since the values for this fraction were very low they were not taken into consideration while calculating the value for insoluble hydroxyproline

The details of the fractionation procedure are described in the following

Preparation of various fractions from the homogenate

A sample of the homogenate corresponding to ½ gram of the embryo or of the skin was transferred to a small test tube of 4—5 ml capacity Since some of the rather viscous homogenate remained on the inner walls of the pipette the latter was calibrated by pipetting several samples into small tared beakers and weighing them Thereafter it was possible to take the sample with an accuracy of ± 2 per cent with the exception of the homog nate from 18 and 20 day old embryos in which the error was no doubt considerably greater since the feathers often blocked the pipette To this sample of about 1 ml 1 ml of concentrated hydrochloric acid was added and it was used after hydrolysis for the determination of *total hyd oxyproline total proline and total nitro en*

The rest of the homogenate was allowed to stand for 24 hours at + 2 C with occasional vigorous shaking after which a sample was taken and centrifuged for

Preparation of the samples for fractionation — After the desired incubation time the embryos were removed from the eggs killed by pressure on the neck and freed from the membranes The embryos were then dried gently with filter paper with the exception of embryos up to the 8th day of development which were dried only by rolling them gently on a dry porcelain dish since the filter paper removed some of the skin from these small embryos When dried the embryos were rapidly weighed and in experiments with whole embryos placed in cold (± 0°C) 1 M NaCl solution 2 ml per g of embryo

In the experiments with the skin the embryos after weighing were skinned on ice cooled metal trays Special attention was paid to the complete removal of feathers with forceps and to the removal of fascia and subcutaneous fat The skin samples obtained were rapidly weighed and put into cold 1 M NaCl 2 ml per g of skin Skins of several embryos were pooled to form one sample

The samples were homogenized with an Ultra Turrax homogenizer (Janke and Kunkel KG Staufen) for 20 seconds in tubes kept in ice cooled water A homogenate which could be fairly easily pipetted was obtained from all samples of the skin and of whole embryos up to and including the 16th day of development Embryos aged 18 and 20 days were first cut up with scissors and then homogenized for 20 seconds three times at 2 minute intervals but even so it was rather difficult to take accurate samples of the homogenate with a pipette This was mainly due to the presence of feathers which stuck together and blocked the pipette for no pieces of embryo were recognizable after the treatment Not longer than 20 seconds was used for the homogenization lest warming of the sample should occur The sample obtained from 2 ¹ day old embryos was too small for the Ultra Turrax homogenizer and it was therefore treated in a homogenizer of Potter Elvehjem type

After careful shaking a sample was taken from the homogenate for dry weight determination The rest of the homogenate was poured with shaking into test tubes which were stoppered and stored at + 2 C

FRACTIONATION PROCEDURES

The procedure used for the preparation of the various hydroxyproline containing fractions was based on the fact that in addition to soluble collagen the neutral salt extracts of connective tissue also contain free amino acids and peptides Therefore it was obvious that after precipitation of the protein bound hydroxyproline of the neutral salt extract the soluble fraction would still contain free amino acids and peptides

In the present procedure 1 M sodium chloride was used for the extraction of the soluble fraction as suggested by LEVENE and GROSS (1959) Proteins of the soluble fraction were then precipitated by addition of cold ethanol to a final concentration of 80 per cent The ethanol soluble fraction was used for the determination of free hydroxyproline and after hydrolysis for the joint determination of free and peptide hydroxyproline Peptide hydroxyproline was then calculated as the difference between the values obtained before and after hydrolysis of the ethanol soluble fraction The precipitate

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The rest of the homogenate was allowed to stand for 24 hours at + 2 C with occasional vigorous shaking after which a sample was taken and centrifuged for

30 min at 30 000 rpm (about 60 000 × g) in a Spinco model L preparative ultracentrifuge in rotor No 40 The clear supernatant between the precipitate and the fat at the surface was drawn off with a narrow tipped pipette

The precipitate was discarded in most cases but sometimes it was resuspended in cold 1 M sodium chloride and recentrifuged The washing was discarded and the precipitate washed once more with 1 M sodium chloride and recentrifuged and thereafter transferred to a small test tube and dried at + 102°C 2—5 ml of 6 N HCl was added to the sample which was used after hydrolysis for the determination of *insoluble hydroxyproline*

Proteins of the supernatant obtained after centrifugation at 30 000 rpm were precipitated by addition of 4 vols of cold ethanol in a centrifuge tube Since 96 vol % ethanol was often used the final concentration of ethanol varied between 77 and 80 vol % The tube was allowed to stand stoppered at + 2 C for 3 hours with occasional stirring after which it was centrifuged at 3 200 rpm in a bench centrifuge (M Christ Osterode Typ U J 1) for 10 minutes

The ethanol soluble supernatant was divided into two parts which were evaporated to dryness on a steam bath in porcelain dishes The parts are here termed sample *a* and *b*

Sample *a* was taken up in a suitable volume of distilled water and filtered through a filter paper (No 589³ Schleicher and Schull Dassel) If the filtrate was not quite clear it was refiltered whereupon a clear filtrate was obtained The filtrate was used for the determination of *free hydroxyproline free proline and free amino nitrogen*

Sample *b* was suspended in 1 ml of 6 N HCl and poured into a small test tube The porcelain dish was washed once with 0 5 ml and twice with 0 25 ml of 6 N HCl and these portions were also poured into the same test tube After hydrolysis sample *b* was used for the determination of the amount of free and peptide hydroxyproline combined and *peptide hydroxyproline* was calculated as *b* — *a*

Since the values for peptide hydroxyproline were very low compared with those for free hydroxyproline the calculation probably gave rather large errors for the peptide hydroxyproline Thus the relatively high standard deviations in peptide hydroxyproline in Table 5 (p 41) are evidently largely due to errors in the analysis In a few embryos negative values were even obtained These values were treated as negative ones when the means and standard deviations were calculated because it is probable that the values for some other embryos were too high Therefore the standard deviations sometimes indicate a dispersion of the values beyond zero The values for peptide hydroxyproline were not determined in all series In such cases the mean values for the whole material in Table 5 (p 41) were calculated from the series in which peptide hydroxyproline had been determined

The ethanol insoluble precipitate was washed with 80 per cent ethanol by resuspension and recentrifugation and transferred to a small test tube The sample was dried at + 102°C and used after hydrolysis with 2 ml of 6 N HCl for the determination of *1 M sodium chloride soluble collagen hydroxyproline*

The citrate soluble collagen hydroxyproline was only determined in a few cases In these experiments the precipitate that was obtained from the homogenate after centrifugation at 30 000 rpm was washed twice by resuspension in cold 1 M NaCl and recentrifugation at 30 000 rpm for 30 min after which the precipitate was suspended in 0 2 M citrate buffer pH 3 C 2 ml per g original wet weight of the tissue taken for this sample and extracted for 24 hours with occasional vigorous shaking

The tube was then centrifuged at 30 000 rpm for 30 min and 1 ml of the citrate soluble supernatant was transferred to a small test tube 1 ml of concentrated HCl was added and the sample used after hydrolysis for the determination of *citrate soluble colla en hydroxyproline*

Since the dry weight of the embryos ranged from 50 to 720 mg/g wet weight the water content of 1 g is about 0 8—0 95 ml Since 2 ml of 1 M NaCl was used per g of fresh tissue about 2 9 ml of the 1 M NaCl soluble supernatant would represent 1 g of fresh tissue Therefore in the calculation 2 9 ml of supernatant was taken to be equal to 1 g of original fresh tissue when the values were converted to values per g wet weight Since 4 vols of ethanol was add d to 1 vol of the 1 M NaCl soluble supernatant 14 5 ml of ethanol soluble supernatant is equal to 1 g of fresh tissue The values for the citrate soluble fraction could not be converted quite accurately to values per g of fresh tissue since some of the 1 M NaCl solution always remained in the precipitate and thus diluted the add d citrate buffer This dilution was not taken into consid ration and it was assumed that 2 ml of citrate s lubl fraction repre ents 1 g of fresh tissue

Hydrolysis and neutrali ation

The small test tubes containing the samples to be hydroly ed were drawn out into ampoules and hydrolysis of the peptide fraction was carried out in an autoclave at 130 C for 3 h and of other fractions at 138 C for 6 h

The effect of hydrolysis was tested by hydrolysing samples taken from a pooled homogenate of 14 day old embryos for 1 5 3 6 and 9 h at 138 C 4 samples in each batch The hydroly es for 6 h and 9 h gave identical values for total hydroxyproline The mean value of the samples hydrolysed for 1 5 h was 93 per cent of this value and of tho e hydrolysed for 3 h 95 per cent In the ethanol soluble fraction hydrolysis for 3 h at 130 C was sufficient to give maximum values

After hydrolysis the ampoules were opened their contents tran ferre l to graduated test tubes and the ampoules washed 3 times with 1—2 ml of distilled water The samples were then neutralized first with 3 N and then with 0 3 N sodium hydroxide using universal indicator papers (E Merck AG Darmstadt) to pH s of 4—6 and made up to a known volume with distilled water After filtration through a filter paper (Schleicher and Schull No 589[2]) the sampl s were stored frozen unless analysed the same day

In the method of PROCKOP and UDENFRIEND (1960) precipitation of humin and neutralization with potassium hydroxide are recommended It was found however that in the present study humin precipitation was unnecessary except in the samples of hydrolysed egg yolk and white and that only in 2 1 3 and 4 day old embryos was it better to u e potassium hydroxide in place of sodium hydroxid for neutralization

Fractionation procedures in isotope experiments

The procedures were essentially the same as already described Additional purification was carried out as follows

The 1 M NaCl insoluble fraction was washed three times by resuspension in 1 M NaCl and recentrifugation at 30 000 rpm for 30 min The portions of 1 M NaCl used

for washing were discarded These washings were followed by suspension in cold distilled water and centrifugation at 30 000 rpm for 30 min The precipitate was transferred to a test tube of 7—8 ml capacity and extracted twice with absolute ethanol and twice with ether always by suspension and centrifugation at 1 500 rpm for 10 min The samples were then dried at 102°C and hydrolysed with 3 ml of 6 N HCl at 138°C for 6 hours

The 1 M NaCl soluble ethanol insoluble fraction was washed twice with 80 per cent ethanol twice with absolute ethanol twice with ether and after drying at 102°C hydrolysed with 3 ml of 6 N HCl at 138 C for 6 hours

After hydrolysis the contents of the test tubes of both hydrolysed fractions were diluted by addition of 3 ml of distilled water and the humins were removed by the method of PROCKOP and UDENFRIEND (1960) The samples were filtered and the filter papers washed several times with small volumes of 2 N HCl The filtrates were evaporated to dryness on a steam bath in porcelain dishes and dissolved in a known volume of distilled water The samples were used for the determination of the specific activities of insoluble and 1 M sodium chloride soluble collagen hydroxyproline

The ethanol soluble fraction was not subjected to more purification procedures than in the other experiments and it was used without hydrolysis for determination of the specific acitivity of free hydroxyproline

RELIABILITY OF THE FRACTIONATION METHOD

The following experiments were carried out to test the reliability of the fractionation method used in the present investigation

1 Crude homogenates of five 15-day old embryos were each divided into two parts One part of each homogenate was fractionated as described under the fractionation procedures The other part of each homogenate received an *addition of 200 μg of hydroxyproline per 3 g wet weight of the embryo* and was then fractionated as above A recovery of 95 to 101 per cent was obtained for the added hydroxyproline when determined as free hydroxy*proline*

2 Since the values for peptide hydroxyproline obtained in the present investigation were low, it seemed necessary to test whether the recovery of hydroxyproline added to the ethanol-soluble fraction was the same in the samples analysed after hydrolysis as in the samples analysed without hydrolysis In this experiment ethanol soluble fractions obtained from four 13 day old embryos were pooled, and the resulting extract divided into 24 equal parts

— Four samples were analysed for hydroxyproline without hydrolysis and four samples after hydrolysis

— Eight samples *received an addition of 20 μg of hydroxyproline per g* wet weight of the embryo Four samples were then analysed without hydrolysis and four samples after hydrolysis

— Eight samples received an addition of 40 μg of hydroxyproline per g wet weight of the embryo and were analysed as above

The results (Table 1) indicated that the recovery of hydroxyproline in the ethanol soluble fraction was the same whether the analysis was made after hydrolysis or without it

3 The value for hydroxyproline present in the unfractionated 1 M sodium chloride-soluble fraction (d) was determined after hydrolysis in samples of this fraction from four 13-day-old embryos The rest of the 1 M sodium chloride soluble fraction was fractionated further and analysed for free (a) peptide (b) and 1 M sodium chloride-soluble collagen hydroxyproline (c) The results (Table 2) indicated that the value obtained by summation of a + b + c was in good agreement with the value d This experiment indicated that significant losses of hydroxyproline do not occur during fractionation of the 1 M sodium chloride soluble fraction

Table 1 Recoveries of hydroxyproline added to the ethanol soluble fraction when analysed without and after hydrolysis

Hydroxyproline added[1]	Hydroxyproline found[1, 2]	
	Without hydrolysis	After hydrolysis
None	54 1 ± 1 1	40 5 ± 0 1
20 $\mu g/g$	75 1 ± 1 4	80 ± 1 1
40 $\mu g/g$	93 6 ± 0 7	100 3 ± 1 0

[1] Expressed as $\mu g/g$ wet weight of the embryo
[2] Each value is mean ± S D of 4 samples

Table Comparison of the values for hydroxyproline in the 1 M sodium chloride soluble fraction obtained by calculation from the values for free (a) peptide (b) and 1 M sodium chloride soluble collagen (c) hydroxyproline and by analysis of an aliquot of the crude 1 M sodium chloride soluble fraction (d)

No	Hydroxyproline[1]				
	a	b	c	a + b + c	d
1	51 8	6 2	67 4	125 4	123 0
2	61 8	4 5	64 1	130 4	137 8
3	53 8	3 9	63 3	121 0	126 1
4	56 0	8 [illegible]	63 7	127 9	130 4

[1] Expressed as $\mu g/g$ wet weight of the embryo

for washing were discarded These washings were followed by suspension in cold distilled water and centrifugation at 30 000 rpm for 30 min The precipitate was transferred to a test tube of 7—8 ml capacity and extracted twice with absolute ethanol and twice with ether always by suspension and centrifugation at 1 500 rpm for 10 min The samples were then dried at 102°C and hydrolysed with 3 ml of 6 N HCl at 138°C for 6 hours

The 1 M NaCl soluble ethanol insoluble fraction was washed twice with 80 per cent ethanol twice with absolute ethanol twice with ether and after drying at 102 C hydrolysed with 3 ml of 6 N HCl at 138 C for 6 hours

After hydrolysis the contents of the test tubes of both hydrolysed fractions were diluted by addition of 3 ml of distilled water and the humins were removed by the method of PROCKOP and UDENFRIEND (1960) The samples were filtered and the filter papers washed several times with small volumes of 2 N HCl The filtrates were evaporated to dryness on a steam bath in porcelain dishes and dissolved in a known volume of distilled water The samples were used for the determination of the specific activities of insoluble and 1 M sodium chloride soluble collagen hydroxyproline

The ethanol soluble fraction was not subjected to more purification procedures than in the other experiments and it was used without hydrolysis for determination of the specific activity of free hydroxyproline

RELIABILITY OF THE FRACTIONATION METHOD

The following experiments were carried out to test the reliability of the fractionation method used in the present investigation

1 Crude homogenates of five 15-day-old embryos were each divided into two parts One part of each homogenate was fractionated as described under the fractionation procedures The other part of each homogenate received an addition of 200 μg of hydroxyproline per 3 g wet weight of the embryo and was then fractionated as above A recovery of 95 to 101 per cent was obtained for the added hydroxyproline, when determined as free hydroxyproline

2 Since the values for peptide hydroxyproline obtained in the present investigation were low it seemed necessary to test whether the recovery of hydroxyproline added to the ethanol-soluble fraction was the same in the samples analysed after hydrolysis as in the samples analysed without hydrolysis In this experiment ethanol soluble fractions obtained from four 13-day old embryos were pooled and the resulting extract divided into 24 equal parts

— Four samples were analysed for hydroxyproline without hydrolysis and four samples after hydrolysis

— Eight samples received an addition of 20 μg of hydroxyproline per g wet weight of the embryo Four samples were then analysed without hydrolysis and four samples after hydrolysis

fied method of KIVIRIKKO and LIESMAA (1959) was used. The values for free hydroxyproline obtained in these experiments are in good agreement.

6. In some experiments embryos were homogenized in 0.15 M sodium chloride solution. The proteins of the homogenate were precipitated in these experiments with trichloroacetic acid. The values for free hydroxyproline obtained were in good agreement with those obtained in the experiments in which 1 M sodium chloride was used. These results indicated that the ionic strength of the sodium chloride solution has no effect on the amount of free hydroxyproline extracted.

The results of the experiments described above indicate that the fractionation method used in the present investigation can be regarded as satisfactory since there is no loss of hydroxyproline (1—4) or artificial formation of free hydroxyproline (5) during the procedure. In addition, the amount of free hydroxyproline extracted is not dependent on the ionic strength of the sodium chloride solution used (6).

4 The value for insoluble hydroxyproline which was generally calculated from the other values was actually determined in some of the experiments The value obtained by analysis always agreed with that calculated to within ± 5 per cent The only exceptions were the embryos of 2[1] and 3 days of age in which the values for total hydroxyproline obtained by analysis were about 10 per cent higher than the calculated values Since the error in these embryos is probably due to too low recoveries in more than one fraction the value obtained by analysis in these embryos was taken as the insoluble fraction and the sum obtained by calculation as the total hydroxyproline This calculation had the advantage that the values for hydroxyproline in the various fractions expressed as a percentage of the total hydroxyproline were probably more correct than if they had been calculated from the value obtained by analysis of total hydroxyproline

5 Crude homogenates of six 13-day old embryos were divided into two parts immediately after homogenization The proteins of one part of each homogenate were precipitated immediately with trichloroacetic acid and after filtration the trichloroacetic acid was removed from the filtrate by three extractions with ether The other part of each homogenate was treated as described in the section on fractionation procedures The values for free hydroxyproline obtained with both procedures are given in Table 3 This experiment indicated that no artificial formation of free hydroxyproline occurs during the fractionation procedure used in the present investigation It is further noteworthy that in this experiment free hydroxyproline was determined by the method of PROCKOP and UDENFRIEND (1960) in contrast to the experiments presented in tables 1 and 2 in which the modi

Table 3 Comparison of the values for free hydroxyproline obtained by trichloroacetic acid precipitation of the homogenate (TCA precipitation) or by the procedure used in the present investigation (Present procedure)

No	Free hydroxyproline [1]	
	TCA precipitation	Present procedure
1	50 3	49 1
2	68 2	71 2
3	51 2	52 8
4	57 0	57 8
5	46 5	48 2
6	48 3	50 2
Mean	53 6	54 9

[1] Expressed as μg/g wet weight of the embryo

Table 4 Numbers wet weights and relative dry weights of the embryos in different series of experiments with whole embryos

Age in days	Series	Number of embryos[2]	Wet weight g ± S D[3]	Dry weight mg/g wet wt. ± S D[3]
2½	J	1 × 194	0 006	60
3	F	1 × 100	0 015	49
	H	1 × 120	0 011	61
	Mean		0 013	55
4	E	1 × 48	0 055	64
5	D	2 × 18	0 159 ± 0 008	66 ± 3
6	C	3 × 12	0 309 ± 0 029	66 ± 2
8	C	3 × 5	1 08 ± 0 06	68 ± 2
10	A	3 × 2	2 39 ± 0 30	73 ± 1
	G	4	2 40 ± 0 18	68 ± 3
	Mean		2 39 ± 0 21	70 ± 4
11	A[1]	6	3 34 ± 0 36	83 ± 2
	B	6	3 33 ± 0 25	77 ± 2
	Mean		3 33 ± 0 30	80 ± 4
12	A	5	4 76 ± 0 38	88 ± 2
13	E	4	7 40 ± 0 60	100 ± 4
14	A	6	7 70 ± 0 92	112 ± 7
	B[1]	4	9 11 ± 0 18	120 ± 8
	I	6	8 68 ± 0 89	104 ± 4
	Mean		8 42 ± 0 96	111 ± 9
16	A	6	13 06 ± 0 65	167 ± 6
	B[1]	6	14 27 ± 0 59	164 ± 6
	C	8	13 40 ± 1 51	167 ± 9
	Mean		13 55 ± 1 14	162 ± 7
18	A	6	16 58 ± 0 84	195 ± 19
	B[1]	[illegible]	17 39 ± 1 25	215 ± 8
	Mean		16 95 ± 1 08	204 ± 17
20	A	5	23 50 ± 1 01	195 ± 18

[1] Received one or more injections of 0 1 ml 0 9 per cent sodium chloride
[2] Number of samples × embryos in sample
[3] In 5-day-old embryos ± deviation

HYDROXYPROLINE-CONTAINING FRACTIONS IN NORMAL CHICK EMBRYOS

The purpose of this part of the investigation was to study the changes occurring in the contents of the various hydroxyproline-containing fractions during the formation of collagen in whole embryos and in the skin of the embryos. This part of the investigation also includes determinations of the specific activities of hydroxyproline in some of the fractions after administration of ^{14}C-proline.

CONTENT OF HYDROXYPROLINE IN THE FRACTIONS OF WHOLE EMBRYOS

Ten different incubation series, designated as series A—J, were made. The values in the tables are mean values ± standard deviations for the age groups of the different incubation series. Since the differences between the different series were relatively small, the pooled mean values ± standard deviations were also calculated for each age group from the whole of the data and these values are presented in the figures. Some groups of embryos in the series A, B and C were used as controls in the experiments in which the effect of cortisone was investigated. These embryos received one or more injections of 0.9 per cent sodium chloride solution. Since these embryos did not differ from the normal ones, they are included in the normal material. However, these groups are indicated in the tables.

Weights of the embryos. — The number of embryos in each series, their wet weights and relative dry weights as mg dry substance per g wet weight are given in Table 4. The weights of the embryos are in fairly good agreement with those presented in other investigations (see ROMANOFF 1960, p. 1141) with the exception of the values for 18- and 20-day-old embryos, which are slightly lower than has been reported by others. Nevertheless, the values obtained by CHVAPIL (1959), for instance, for 20-day-old embryos were of the same magnitude as in the present investigation.

Table 4 Numbers, wet weights and relative dry weights of the embryos in different series of experiments with whole embryos

Age in days	Series	Number of embryos[2]	Wet weight g ± SD[3]	Dry weight mg/g wet wt ± SD[3]
2	J	1 × 194	0.006	60
3	F	1 × 100	0.015	49
	H	1 × 120	0.011	61
	Mean		0.013	55
4	E	1 × 48	0.055	64
5	D	2 × 18	0.159 ± 0.008	66 ± 3
6	C	3 × 12	0.309 ± 0.029	66 ± 2
8	C	3 × 5	1.08 ± 0.06	68 ± 2
10	A	3 × 2	2.39 ± 0.30	73 ± 1
	G	4	2.40 ± 0.18	68 ± 3
	Mean		2.39 ± 0.21	70 ± 4
11	A[1]	6	3.31 ± 0.36	83 ± 2
	B	6	3.33 ± 0.25	77 ± 2
	Mean		3.33 ± 0.30	80 ± 4
12	A	5	4.76 ± 0.38	88 ± 2
13	E	4	7.40 ± 0.60	100 ± 4
14	A[1]	6	7.70 ± 0.92	112 ± 7
	B	4	9.11 ± 0.18	120 ± 8
	I	6	8.68 ± 0.89	104 ± 4
	Mean		8.42 ± 0.96	111 ± 9
16	A	6	13.06 ± 0.65	162 ± 6
	B[1]	6	14.27 ± 0.59	164 ± 6
	C[1]	8	13.40 ± 1.51	162 ± 9
	Mean		13.55 ± 1.14	162 ± [illegible]
18	A	6	16.58 ± 0.84	195 ± 19
	B	5	17.39 ± 1.25	215 ± 8
	Mean		16.9 ± 1.08	204 ± 17
20	A	5	23.50 ± 1.01	195 ± 18

[1] Received one or more injections of 0.1 ml 0.9 per cent sodium chloride
[2] Number of samples × embryos in sample
[3] In 5-day-old embryos ± deviation

CHANGES IN THE CONTENTS OF FREE, PEPTIDE, 1 M SODIUM CHLORIDE SOLUBLE COLLAGEN, CITRATE SOLUBLE COLLAGEN, INSOLUBLE AND TOTAL HYDROXYPROLINE DURING EMBRYONIC DEVELOPMENT

The values for hydroxyproline are presented mainly as µg per 100 mg dry weight of the embryo and as a percentage of the total hydroxyproline. In some cases, when the values expressed as µg per g wet weight or as µg per whole embryo are of interest, these values are mentioned in the text. A short preliminary report of a part of the results described in this section has already been published (KIVIRIKKO 1963 b).

The contents of free, peptide, 1 M sodium chloride soluble collagen, insoluble and total hydroxyproline in the various series and in the whole material are given in Table 5. The reproducibility of the values was rather high. Since, moreover, the standard deviations are mostly relatively small, it appears that the hydroxyproline contents at a given stage of development were relatively constant. Some of the variation in the values depends upon the variation in the weights of the embryos. However, when the hydroxyproline values were plotted against the weights of the embryos, the scatter was not essentially smaller, indicating that it was not solely due to weight differences. Therefore the values are hereafter only expressed as a function of the ages of the embryos.

All the fractions presented in Table 5 were already present in the embryo at the age of 2 ½ days. In all fractions the absorption spectra obtained for the coloured compound in the method of PROCKOP and UDENFRIEND (1960) showed the characteristic peak at 560 mµ. Therefore the content of hydroxyproline was also determined in hydrolysed samples of crude egg yolk and white. In both samples some colour was obtained at 560 mµ, but the absorbances were only slightly higher at 560 mµ than at 500 mµ. If all the colour obtained had been derived from hydroxyproline, the values would have been about 2 µg/100 mg dry matter for egg yolk and about 5 µg/100 mg dry matter for egg white. The correct values must be considerably lower, which indicates that even at the age of 2 ½ days most, if not all, of the hydroxyproline was newly synthesized. Smaller embryos could not be obtained in amounts sufficient for the fractionation procedure.

The contents of free, peptide, 1 M sodium chloride soluble collagen, insoluble and total hydroxyproline expressed as µg/100 mg dry weight of the embryo and the absolute amounts of dry matter per embryo are also presented in Fig. 2 (upper and lower).

The contents of *total* and *insoluble hydroxyproline* increased continuously during embryonic development. This increase was about 27 fold in total hydroxyproline and about 18 fold in insoluble hydroxyproline.

Table 5 Hydroxyproline-containing fractions in whole embryos in different series and in the material as a whole

Age in days	Series	Hydroxyproline* Free	Peptide	1 M NaCl soluble collagen	Insoluble	Total
2½	J	6.8	1.2	11.9	20.3	39.5
3	F	10.2	1.0	18.6	37.7	67.5
	H	9.2	1.0	13.8	25.2	49.2
	Mean	9.7	1.0	16.2	31.5	58.4
4	F	1?.4	1.?	14.7	35.6	?3.9
5	D	20.9 ± 0.3	1.8 ± 0.2	18.9 ± 0.8	57.5 ± 2.6	99.1 ± 3.9
6	C	25.7 ± 1.3	1.4 ± 0.2	20.5 ± 1.1	78.4 ± 6.6	126 ± 8
8	C	23.2 ± 1.1	2.3 ± 1.0	31.0 ± 1.6	175 ± 13	231 ± 17
10	A	2?.0 ± 0.4	4.2 ± 0.7	37.8 ± 1.3	283 ± 40	352 ± 46
	G	29.4 ± 1.0	—	41.9 ± 2.1	298 ± 24	340 ± 27
	Mean	28.4 ± 1.5	4.2 ± 0.7	40.1 ± ?.9	292 ± 33	362 ± 34
11	A[1]	33.1 ± 2.9	—	39.4 ± 3.1	3?3 ± 36	445 ± 40
	B	35.1 ± 4.7	—	40.5 ± 2.7	424 ± 35	509 ± 39
	Mean	34.1 ± 3.8	—	44.5 ± 6.0	398 ± 43	477 ± 50
12	A[1]	46.4 ± 6.3	3 ± ?.3	50.6 ± 1.8	503 ± 24	604 ± 21
13	E	56.2 ± 4.1	5.? ± ?.0	65.0 ± 1.9	558 ± 20	685 ± 21
14	A	62.7 ± 4.4	4.5 ± 6.0	65.4 ± ?.?	637 ± 52	740 ± 47
	B[1]	55.5 ± 7.7	—	?2.6 ± 6.7	612 ± 51	?40 ± 62
	I	66.4 ± 6.2	4.0 ± 2.8	69.4 ± 5.1	634 ± 22	?4 ± ?5
	Mean	6?.3 ± ?.0	4.2 ± 4.5	68.7 ± 6.0	630 ± 41	764 ± 44
16	A	38.3 ± 4.0	5.3 ± 4.9	59.3 ± 2.7	723 ± 95	826 ± 95
	B	39.1 ± 5.9	—	67.2 ± 3.8	812 ± 45	915 ± 49
	C	35.2 ± 7.3	—	69.1 ± 4.8	770 ± 93	873 ± 96
	Mean	37.3 ± 6.0	5.3 ± 4.9	65.2 ± 5.?	769 ± 86	873 ± 86
18	A	18.1 ± 2.4	4.6 ± 1.3	61.6 ± 7.2	831 ± 362	915 ± 3?0
	B[1]	18.1 ± 3.0	—	67.4 ± ?.5	—	—
	Mean	18.1 ± ?.6	4.6 ± 1.3	64.3 ± 6.1	831 ± 362	915 ± 370
20	A	16.4 ± 3.3	10.3 ± 2.3	56.4 ± 3.9	971 ± 95	1054 ± 97

[1] Received one or more injections of 0.1 ml 0.9 per cent sodium chloride

* µg/100 mg dry weight of the embryo mean ± standard deviation in 5-day old embryos ± deviation

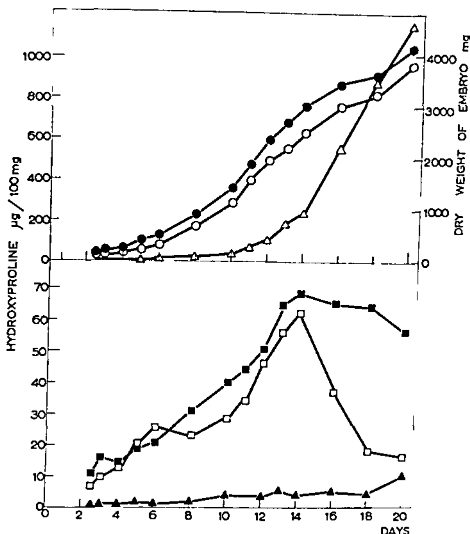


Fig 2 Contents of free □ peptide ▲ 1 M NaCl soluble collagen ■ insoluble ○ and total ● hydroxyproline in whole embryos expressed as μg/100 mg dry weight of the embryo In addition in the upper graph the absolute dry weights of embryos are shown on the ordinate on the right Abscissa Age of embryos in days

The contents of *free and 1 M sodium chloride-soluble collagen hydroxy proline* both increased up to the 14th day of development After this a rapid decrease was observed in the content of free hydroxyproline and a slight decrease in the content of 1 M sodium chloride soluble collagen hydroxy proline If the values are expressed as μg per g wet weight a similar maximum is observed in free hydroxyproline at the age of 14 days but the rate of decrease is then less rapid than in Fig 2 for the following values are obtained 69 1 μg/g in 14- 60 1 μg/g in 16- 37 0 μg/g in 18- and 31 5 μg/g in 20 day old embryos By contrast the content of 1 M sodium chloride

soluble collagen hydroxyproline increases up to the 18th day of development if this value is expressed as $\mu g/g$ wet weight Even if the values for free hydroxyproline are expressed as absolute values in μg/embryo a decrease is observed towards the end of embryonic development although the increase in the weight of the embryos displaces the maximum value to the 16th day The values for free hydroxyproline per embryo are as follows 583 μg in 14 816 μg in 16- 627 μg in 18- and 738 μg in 20-day-old embryos

To show that there is no possibility that the decrease in the content of free hydroxyproline might be due to an unusual delay in the growth curve towards the 18th day of development the values that were obtained in five 18-day old embryos in preliminary experiments are presented In those experiments free hydroxyproline was not determined but the value for ethanol soluble hydroxyproline (free and peptide hydroxyproline) can be compared with the values for free and peptide hydroxyproline of series A At the age of 14 days the mean weight of the embryos was 9 23 and at the age of 18 days 20 85 g in the preliminary experiments The corresponding values for ethanol soluble hydroxyproline were 61 7 μg/100 mg dry weight and 21 6 μg/100 mg dry weight which are in good agreement with the values of series A (cf Table 5)

The content of *peptide hydroxyproline* did not show marked changes during embryonic development A definite increase was observed however when the values obtained in young embryos were compared with those of old embryos In 20-day-old embryos peptide hydroxyproline formed a noteworthy part of the total ethanol soluble hydroxyproline since a decrease in free hydroxyproline and an increase in peptide hydroxyproline occurred during the final stage of embryonic development

The content of *citrate soluble collagen hydroxyproline* was only determined in a few series The following values expressed as μg/100 mg dry weight of the embryo were obtained 0 0 in 4 1 6 in 6- 1 8 in 10- 4 8 in 13- and 4 4 in 16-day old embryos Although the insoluble fraction was washed twice with 1 M sodium chloride after the extraction of the 1 M sodium chloride soluble fraction it is still possible that some of the above values may be derived from 1 M sodium chloride soluble hydroxyproline that has remained in spite of the washing of the insoluble residues The content of citrate soluble collagen hydroxyproline is thus very low in chick embryos

The contents of free peptide 1 M sodium chloride soluble collagen and insoluble hydroxyproline *expressed as a percentage of total hydroxyproline* are presented in Fig 3 (upper and lower) In the youngest embryos the content of insoluble hydroxyproline formed 51 5 per cent of the total hydroxy

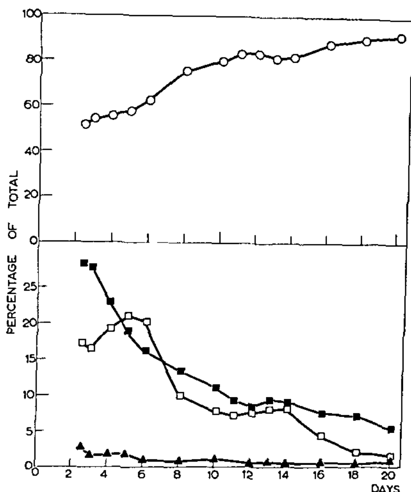


Fig 3 Contents of free □ peptide ▲ 1 M NaCl soluble collagen ■ and insoluble ○ hydroxyproline in whole embryos expressed as a percentage of total hydroxyproline Abscissa Age of embryos in days

proline A great increase in this fraction then occurred during embryonic development but between the 11th and 14th days there was a period with no relative increase in this fraction In 20 day-old embryos insoluble hydroxyproline formed 92 1 per cent of the total hydroxyproline

In contrast to the insoluble hydroxyproline the relative amount of 1 M sodium chloride-soluble collagen hydroxyproline decreased during embryonic development Nevertheless between the 11th and 14th days there was a period during which the relative amount of this fraction showed no appreciable change The curve of free hydroxyproline differs from that of 1 M sodium chloride soluble collagen hydroxyproline only in that a small initial increase occurred between the ages of 2½ and 5 days At the age of 2½ days the value was 17 1 per cent and then varied from 15 1 to 21 1 per

cent until the 5th day of development Thereafter this curve shows a decrease until the 10th day and a further decrease after the 11th day to the value 1 6 per cent in 20-day-old embryos

The percentage of peptide hydroxyproline was small throughout embryonic development and no noteworthy changes occurred in this fraction The relative amount of citrate soluble collagen hydroxyproline which is not presented in the figure was smaller still

COMPARISON OF CHANGES IN HYDROXYPROLINE WITH CHANGES IN FREE AND TOTAL PROLINE FREE AMINO NITROGEN AND TOTAL NITROGEN

The contents of *free proline* and *free amino nitrogen* after the 10th day of development are presented in Fig 4 Between the 10th and 11th days of development at which time there occurred a great increase in the content of free hydroxyproline there was also some increase in the content of free proline but no significant changes in the content of free amino nitrogen After the 11th day when the content of free hydroxyproline showed a rapid decrease there was also a decrease in the contents of free proline and free amino nitrogen The magnitude of these decreases was considerably smaller however than that of free hydroxyproline Since the values for free proline and free amino nitrogen also include some peptides (see p 28) the extents of the changes in these fractions are possibly somewhat erroneous The direction of the changes (decrease after the 11th day) must nevertheless be regarded as significant

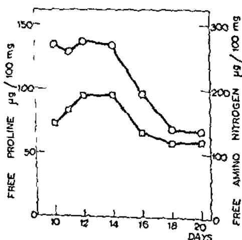


Fig 4 Contents of free proline □ and free amino nitrogen ○ in whole embryos expressed as µg/100 mg dry weight of the embryo Abscissa Age of embryos in days

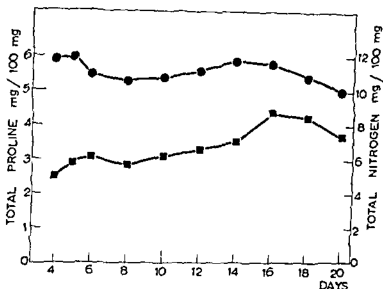


Fig 5 Contents of total proline ■ and total nitrogen ● in whole embryos expressed as mg/100 mg dry weight of the embryo Abscissa Age of embryos in days

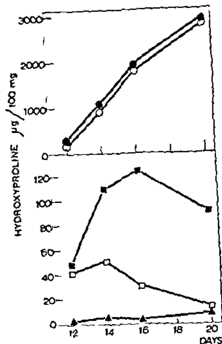
The contents of total proline and *total nitrogen* were determined after the 4th day of development (Fig 5) The content of total proline increased to the 16th day, after which there was some decrease towards the end of incubation period Throughout the observation period the content of total nitrogen was between 10 and 12 mg/100 mg dry weight indicating that the changes in hydroxyproline that were expressed in relation to the dry weight of the embryo would have been essentially similar if they had been expressed in relation to the total nitrogen

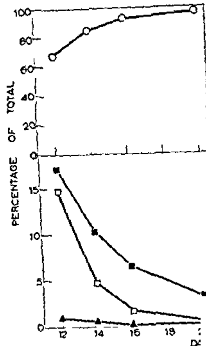
CONTENT OF HYDROXYPROLINE IN THE FRACTIONS OF THE SKIN

To compare whether the changes observed during embryonic development in the contents of the various hydroxyproline-containing fractions in whole embryos are similar to those that occur in tissues rich in collagen the fractions were also analysed in the skin after the 12th day of development

Two series of experiments were made The values for each sample are presented in Table 14 in the Appendix Differences could be observed between the age groups although there were only a few samples in each for the reproducibility of the values seemed to be fairly good The differences in the dry weights of samples of the same age group are probably due to varying degrees of water loss during their preparation

The contents of free peptide 1 M sodium chloride soluble collagen insoluble and total hydroxyproline *expressed as µg/100 mg dry weight of the*





Fi... 6 Contents of free □ peptide ▲ 1 M NaCl soluble collagen ■ insoluble ○ a total ● hydroxyproline in the skin expressed as μg 100 mg dry weight of the sk Abscissa Age of embryos in days

Fig Contents of free □ peptide ▲ 1 M NaCl soluble collagen ■ and insoluble hydroxyproline in the skin expressed as a percentage of the total hydroxyproline Abscis Age of embryos in days

skin are presented in Fig 6 (upper and lower) An increase of about 10-f occurred in the content of total hydroxyproline and of about 14 fold in t content of insoluble hydroxyproline between days 12 and 20 The cont of 1 M sodium chloride-soluble collagen hydroxyproline in the skin w already considerably higher than that of free hydroxyproline at the age 14 days and in the former fraction in the skin the maximum value was reached until the 16th day whereas in whole embryos it occurred at age of 14 days In other respects the curves presented in Fig 6 are v similar to those obtained in whole embryos The maximum value for f hydroxyproline occurred in the skin and in whole embryos at the age 14 days and a decrease then occurred in free hydroxyproline there be an increase in the peptide hydroxyproline towards the end of embry

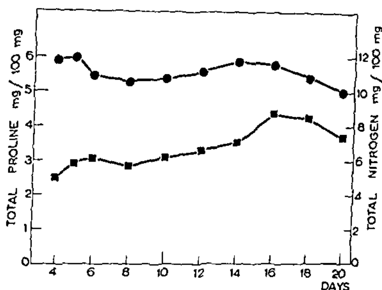


Fig. 5 Contents of total proline ■ and total nitrogen ● in whole embryos expressed as mg/100 mg dry weight of the embryo Abscissa Age of embryos in days

The contents of *total proline* and *total nitrogen* were determined after the 4th day of development (Fig 5) The content of total proline increased to the 16th day, after which there was some decrease towards the end of incubation period Throughout the observation period the content of total nitrogen was between 10 and 12 mg/100 mg dry weight indicating that the changes *in hydroxyproline that* were expressed *in relation to the dry weight of the* embryo would have been essentially similar if they had been expressed in relation to the total nitrogen

CONTENT OF HYDROXYPROLINE IN THE FRACTIONS OF THE SKIN

To compare whether the changes observed during embryonic development in the contents of the various hydroxyproline-containing fractions in whole embryos are similar to those that occur in tissues rich in collagen the fractions were also analysed in the skin after the 12th day of development

Two series of experiments were made The values for each sample are presented in Table 14 in the Appendix Differences could be observed between *the age groups although there were only a few samples in each* for the reproducibility of the values seemed to be fairly good The differences in the dry weights of samples of the same age group are probably due to varying degrees of water loss during their preparation

The contents of free peptide 1 M sodium chloride-soluble collagen insoluble and total hydroxyproline *expressed as μg/100 mg dry weight of the*

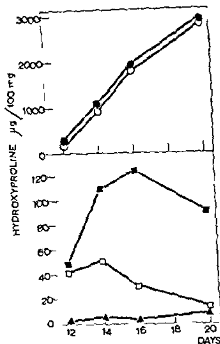


Fig 6

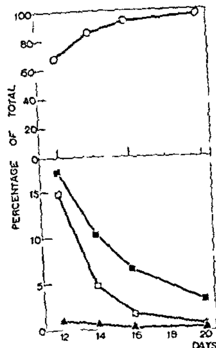


Fig 7

Fig 6 Contents of free □ peptide ▲ 1 M NaCl-soluble collagen ■ insoluble ○ and total ● hydroxyproline in the skin expressed as μg/100 mg dry weight of the skin Abscissa Age of embryos in days

Fig 7 Contents of free □ peptide ▲ 1 M NaCl-soluble collagen ■ and insoluble ○ hydroxyproline in the skin expressed as a percentage of the total hydroxyproline Abscissa Age of embryos in days

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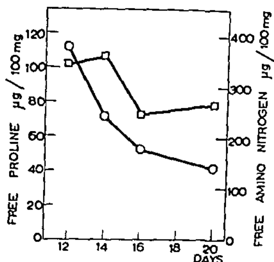


Fig 8

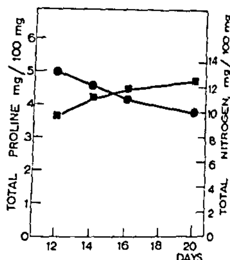


Fig 9

Fig 8 Contents of free proline □ and free amino nitrogen ○ in the skin expressed as µg/100 mg dry weight of the skin Abscissa Age of embryos in days

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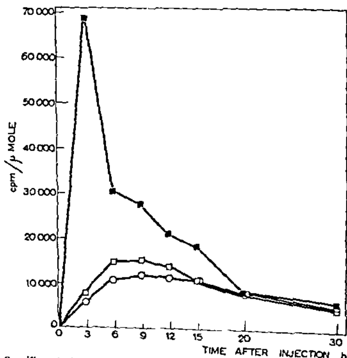


Fig 10 Specific activities of free □ 1 M NaCl soluble collagen ■ and insoluble ○ hydroxyproline after the administration of 2 μC of ^{14}C-proline to 11 day-old chick embryos

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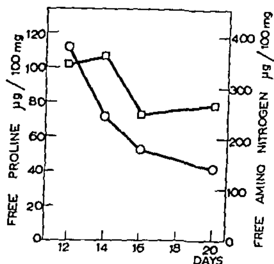


Fig 8

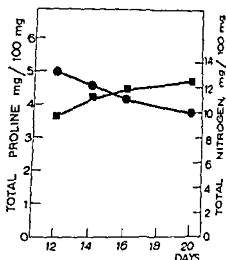


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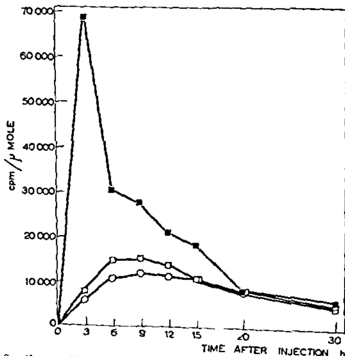


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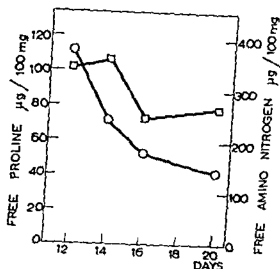


Fig 8

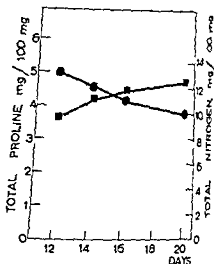


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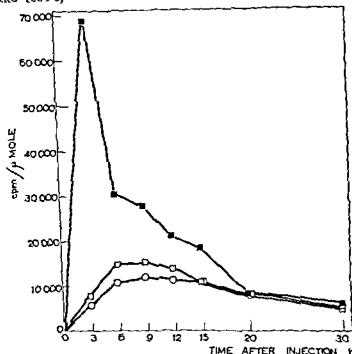


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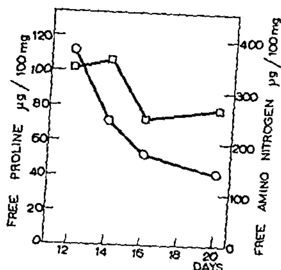


Fig 8

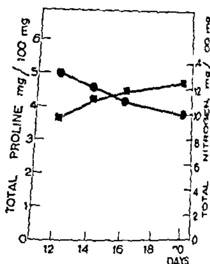


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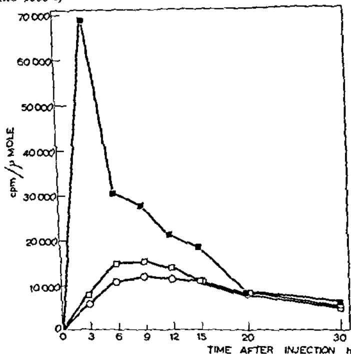


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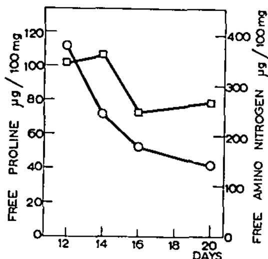


Fig 8

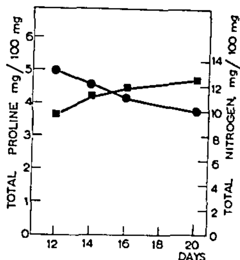


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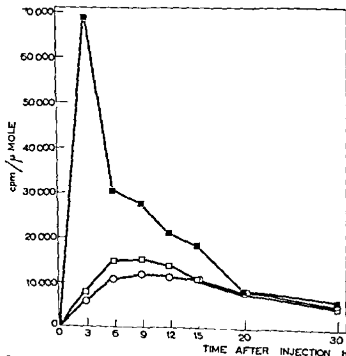


Fig. 10. Specific activities of free □, 1 M NaCl soluble collagen ■ and insoluble ○ hydroxyproline after the administration of 2 μC of ^{14}C-proline to 11 day old chick embryos.

Two series of experiments were made. In both series 2 μC of uniformly labelled L-^{14}C proline (10.8 μC/μmole, The Radiochemical Centre, Amersham) was injected in 100 μl of 0.9 per cent sodium chloride solution onto the chorioallantoic membrane of 11-day-old embryos. The values for each embryo are presented in Table 15 in the Appendix and the mean values for each group in Fig. 10. There was some variation between the values of the same group, but this variation was partly due to weight differences at the time of isotope administration.

The specific activity of the *hydroxyproline of the 1 M sodium chloride-soluble collagen* fraction reached its maximum value at 3 h after which there was a continuous decrease during the observation period The rate of this decrease was considerably more rapid between 3 and 6 h than thereafter The ratio of the specific activity of 1 M sodium chloride soluble collagen hydroxyproline to the specific activity of insoluble hydroxyproline calculated from the mean values of the groups was 11 8 at 3 h 2 78 at 6 h 2 29 at 9 h, 1 85 at 12 h 1 65 at 15 h 1 06 at 20 h and 1 02 at 30 h

The specific activity of *free hydroxyproline* was considerably less than that of 1 M sodium chloride soluble collagen hydroxyproline during the first 15 hours but slightly higher than that of insoluble hydroxyproline during the first 12 hours No sharp peak could be observed in the specific activity of free hydroxyproline but the maximum evidently occurred between 6 and 9 h The ratio of the specific activity of free hydroxyproline to the specific activity of insoluble hydroxyproline was 1 31 at 3 h 1 36 at 6 h 1 27 at 9 h 1 22 at 12 h 0 97 at 15 h, 1 03 at 20 h and 0 90 at 30 h

The specific activity of *insoluble hydroxyproline* also increased rapidly and showed a maximum value at as little as 9 h The decrease observed thereafter was mainly due to synthesis of new unlabelled hydroxyproline in the chick embryo This was evident when an attempt was made to calculate the total activities of insoluble hydroxyproline from the amounts of insoluble hydroxyproline per embryo These values were calculated for embryos of different weights from the embryos used in the experiments summarized in Table 5 p 11 The following mean values were obtained for the total activities of insoluble hydroxyproline as cpm/embryo 112 000 at 9 h *117 000 at 12 h 118 000 at 20 h and 115 000 at 30 h* The results indicated that there was no appreciable change in the total activity of insoluble hydroxyproline between *9 and 30 h Since the rates of increase in the contents* of free and insoluble hydroxyproline are similar at this stage of development *increases in their contents cannot be the explanation of the differences in* the rates of decrease in their specific activities that were noted towards the *end of the observation period*

DISCUSSION

Since the rate of turnover of collagen is low in mature connective tissue most investigations dealing with the metabolism of collagen have been carried out with material in which rapid formation of new connective tissue is occurring Much use has been made of the granulomas induced by subcutaneous injection of carrageenin or the connective tissue induced by subcutaneous implantation of polyvinyl sponges During recent years developing

chick embryos have been increasingly used especially in investigations concerning the action of lathyrogenic agents on collagen formation In the present study it was found that the hydroxyproline of chick embryos could be fractionated satisfactorily by the procedure used and that chick embryos seemed to be material giving reproducible results and suitable for the study of collagen formation

NEUMAN (1950) did not find hydroxyproline in chick embryos until the 5th day of development but CHVAPIL (1959) has reported the presence of hydroxyproline even in 4 day-old chick embryos Nor was hydroxyproline found in frog embryos during the earliest stages of development (EDDS 1958) Nothing was known about the fractions of hydroxyproline during its appearance in the embryos In the present investigation all the fractions studied except citrate soluble collagen hydroxyproline were found to be present in the embryos after an incubation time of as little as 2½ days Since the hydroxyproline content was found to be very small in hydrolysed egg yolk and white it seems evident that most if not all of the hydroxyproline already present in the embryos at this stage of development was newly synthesized

The total and insoluble hydroxyproline contents per 100 mg dry weight of the embryo or of the skin increased continuously throughout embryonic development The increase in total hydroxyproline was about 27 fold in whole embryos between the ages of 2½ and 20 days and about 10-fold in the skin between the ages of 12 and 20 days The corresponding values were still greater in the case of the insoluble hydroxyproline In contrast to the total hydroxyproline the content of total proline increased only slightly and the content of total nitrogen did not show noteworthy changes during embryonic development The values observed for total hydroxyproline are in fairly good agreement with those presented by LEVENE and GROSS (1959) for the skin of 14 and 17 day-old embryos and by NEUMAN (1950) and by *CHVAPIL (1959)* for whole embryos with the exception of the youngest Since the method of PROCKOP and UDENFRIEND (1960) has a greater specificity and accuracy than that of NEUMAN and LOGAN *(1950) used in the* earlier investigations the present values are possibly *more reliable*

Collagen fibres are built up from macromolecules called *tropocollagen* which are synthesized within the fibroblasts and from there transported into the extracellular space where they aggregate together to form collagen fibres (see p 9) During the maturation of *connective tissue these* fibres increase in amount and the relative amounts of cellular and extracellular non fibrous *elements decrease* Therefore the increase observed during embryonic development in the *present study* in the relative amount of insoluble hydroxyproline expressed as a percentage of the total hydroxy

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Earlier investigations had already indicated that there are relatively large amounts of free hydroxyproline in chick embryos (*e g* ROBERTS KARNOFSKY and FRANKEL 1951 PASIEKA and MORGAN 1956 DECKER LEVENE and GROSS 1959 MITOMA SMITH FRIEDBERG and RAYFORD 1959 CHVAPIL 1959) but the values presented were somewhat variable The observation of CHVAPIL (1959) that the value for free hydroxyproline in the whole embryo was about 20 to 30 μg/100 mg dry weight between the 8th and 20th days of development, could not be confirmed MITOMA *et al* (1959) reported a value of 300 μg free hydroxyproline per 13-day-old embryo which also suggests a higher concentration at the age of 13 days than that observed by CHVAPIL (1959)

Recently studies have been made on the changes occurring in the ultrafiltrable *i e* free and peptide hydroxyproline during the formation of collagen in the carrageenin granuloma (CHVAPIL and ČMUCHALOVÁ 1960 1961) and in free hydroxyproline during the formation of collagen in polyvinyl sponge implant connective tissue (WOESSNER and BOUCEK 1961) These investigations indicated that the maximum content of ultrafiltrable hydroxyproline in the carrageenin granuloma and of free hydroxyproline in polyvinyl sponge implant connective tissue was reached at the same stage as the maximum value of deoxyribonucleic acids which reflects the number of cells Further the bulk of the hydroxyproline in the microsomal fraction of the carrageenin granuloma was ultrafiltrable (CHVAPIL HOLEČKOVÁ ČMUCHALOVÁ KOBRLE and HURYCH 1962) These findings suggested that free and peptide hydroxyproline participate in the synthesis of collagen and are intracellular although their origin from breakdown could not be excluded (CHVAPIL and ČMUCHALOVÁ 1961 CHVAPIL *et al* 1962) WOESSNER and BOUCEK (1961) also suggested that there may normally be a constant amount of free hydroxyproline within the fibroblasts

In the present investigation the values for free hydroxyproline and 1 M sodium chloride soluble collagen hydroxyproline resembled each other in many respects Both fractions showed a maximum value at the age of 14 days expressed per 100 mg dry weight and both fractions formed a considerable part of the total hydroxyproline in the early stages of embryonic development The similarities between the changes in the free and 1 M sodium chloride soluble collagen hydroxyproline and the fact that there were no similarities between the changes in free hydroxyproline and insoluble hydroxyproline suggest that free hydroxyproline in chick embryos might be derived to a great extent from the 1 M sodium chloride-soluble collagen hydroxyproline Experiments with ^{14}C-proline in the present investigation provide evidence in support of this suggestion and opposing such possibilities as that free hydroxyproline in chick embryos is derived in considerable

proline was expected for this fraction is mainly derived from the insoluble collagen fibres. The decrease during embryonic development in the 1 M sodium chloride-soluble collagen hydroxyproline expressed as a percentage of total hydroxyproline was likewise expected as this fraction represents the intracellular and extracellular non aggregated collagen molecules with some additional collagen from the surface of the fibres (pp. 9—10).

Changes in relative amounts similar to those occurring in the above fractions have been described by JACKSON (1957) during the first days of development of the carrageenin granuloma where likewise an initial decrease in the relative amount of neutral salt-soluble collagen and an increase in the relative amount of insoluble collagen were seen. In the carrageenin granuloma 60 per cent of the total hydroxyproline was insoluble after 3 days development but JACKSON (1957) considered it possible that this insoluble collagen was derived from contamination with collagen from pre-existing loose fascia and did not represent new insoluble collagen. In the present investigation in which the insoluble hydroxyproline formed 51.5 per cent of the total hydroxyproline after incubation for 2½ days such contamination seems impossible since the embryos could be obtained free from the shell membranes. Some contamination from the embryonic membranes is certainly possible but these membranes are not present before incubation and thus the insoluble hydroxyproline found after incubation for 2½ days must be newly synthesized.

However, the insoluble hydroxyproline present in young embryos does not necessarily represent the extracellular insoluble collagen fibrils. LOWTHER, GREEN and CHAPMAN (1961) have found that in the carrageenin granuloma only 80 per cent of the microsomal collagen and only 35 per cent of the mitochondrial collagen could be extracted with 0.2 M sodium chloride. An additional 15 to 20 per cent of the mitochondrial collagen could be extracted with 0.45 M sodium chloride but about half the mitochondrial collagen still remained unextracted. In the light of their investigation it is possible that in the young chick embryos a considerable amount of the insoluble hydroxyproline might be derived from intracellular insoluble collagen.

The content of free hydroxyproline also showed marked changes during embryonic development. At the age of 2½ days free hydroxyproline amounted to 6.8 μg/100 mg dry weight and thereafter an increase occurred to a maximum value of 62.3 μg/100 mg at the age of 11 days. This stage was followed by rapid decrease in free hydroxyproline to a value of 16.4 μg/100 mg at the age of 20 days. This decrease was also visible when the values were expressed per unit wet weight and even the absolute amount of free hydroxyproline showed a decrease after the 16th day. A similar maximum was also seen in the free hydroxyproline of the skin at the age of 11 days.

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amounts either from the activated *hydroxyproline* or alternatively from the insoluble hydroxyproline alone (p 57) Studies on the effect of age growth scurvy and lathyrism on the excretion of *hydroxyproline in the* urine also suggest that a considerable part of the hydroxyproline in the urine of young animals is derived from soluble collagens (*e g* ZIFF KIBRICK DREISNER and GRIBLTZ 1956 MARTIN MERGENHAGEN and PROCKOP 1961 JASIN and ZIFF 1962, JASIN FINK SMILEY and ZIFF 1962 JASIN FINK WISE and ZIFF 1962)

Since the 1 M sodium chloride soluble collagen hydroxyproline represents mainly the intracellular and extracellular recently synthesized collagen molecules the above hypothesis that the free hydroxyproline of chick embryos *is largely derived from the 1 M* sodium chloride-soluble collagen hydroxyproline fraction of the present study is in agreement with observations *indicating that the content of free hydroxyproline is related to that of* deoxyribonucleic acids in the carrageenin granuloma and in the polyvinyl sponge implant connective tissue (CHVAPIL and ČMUCHALOVA 1960 1961 WOESSNER and BOUCEK 1961) In addition such a correlation is also found in chick embryos for the maximum content of deoxyribonucleic and ribonucleic acids occurs in chick embryos near the 11th day of development (NOVIKOFF and POTTER 1948 REDDY LOMBARDO and CERECEDO 1952 MARRIAN, HUGHES and WERBA 1956)

The content of peptide hydroxyproline was very low throughout embryonic development Although there was some increase in this fraction, when expressed in relation to dry weight during embryonic development this *increase was so small that there were no noteworthy changes in the relative* amount It is of interest however that a small increase in peptide hydroxyproline was seen towards the end of embryonic development both in whole embryos and in the skin although there was a simultaneous decrease in free hydroxyproline Thus peptide hydroxyproline formed a considerable part of the total ethanol soluble hydroxyproline in 20 day old embryos The values are in agreement with those presented by DECKER LEVINE and GROSS (1959) for acid extractable peptides in 10- and 17 day old embryos

The present data do not allow any definite conclusions to be drawn about the significance *of peptide hydroxyproline in the collagen metabolism* of chick embryos The small amounts present made it difficult to study this fraction with the isotope technique and no such study was therefore made It seems possible that at least some part of the small hydroxyproline containing peptides represents the stage preceding free hydroxyproline in the catabolism of collagen molecules LINDSTEDT and PROCKOP (1961) have found that the specific activities of free and peptide hydroxyproline in the

urine were equal after the administration of ^{14}C-proline but it is not known whether the peptides studied in the present investigation can be compared with the peptides of the urine Recently HURYCH and CHVAPIL (1962 b) have reported that the specific activity of ultrafiltrable hydroxyproline became very high during the incubation of skin slices of 17 day-old chick embryos *in vitro* with ^{14}C-proline In their experiments about half the ultrafiltrable hydroxyproline was free and about half peptide bound but in the present investigation only about 10 to 20 per cent of the ethanol soluble hydroxyproline in the skin of 16-day-old embryos and in whole 16 and 18 day old embryos was peptide-bound It seems probable therefore that the bulk of their ultrafiltrable peptides was present in the 1 M sodium chloride-soluble collagen fraction in the present study Similarly the highly active non dialysable peptide collagen precursor found by LEVINE (1962) during incubation of the skin of young rats *in vitro* might be insoluble in 80 per cent ethanol and therefore the ethanol soluble peptides possibly do not include this fraction

The finding that the content of citrate soluble collagen hydroxyproline was very low both in whole embryos and in the skin is in distinct contrast to the observations in the carrageenin granuloma Certainly the content of citrate soluble collagen is also very low in the carrageenin granuloma on the 3rd day of development but thereafter there is a rapid increase in this fraction (JACKSON 1957) It is also well known that after birth the skin of animals contains considerable amounts of citrate-soluble collagen JACKSON (1957) has suggested that the large amounts of citrate soluble collagen in the carrageenin granuloma may be due to the resorption of collagen that occurs simultaneously with its synthesis in the carrageenin granuloma Values for citrate soluble hydroxyproline in chick embryos similar to those obtained in the present investigation have also been reported by HERRMANN and BARRY (1955) who stated that attempts to extract citrate soluble procollagen were inconclusive and by LEVENE and GROSS (1959) who reported that collagen could not be extracted from bone and skin of 17 day-old normal embryos with acid citrate buffers or with 0 5 per cent acetic acid In the skin of 6 to 8 cm long pig embryos the content of citrate soluble collagen has likewise been found to be negligible but in the 19 cm long embryos this fraction is already present (TUSTANOVSKIJ ZAJDES ORLOVSKAJA and MJAGKAJA 1961) Thus it seems evident that citrate soluble collagen is a fraction of little importance in the formation of embryonic collagen

The results of the present investigation indicated the occurrence of changes of some kind in the metabolism of collagen both in whole embryos and in the skin around the 14th day of development as evidenced by the contents

of free and 1 M sodium chloride-soluble collagen hydroxyproline Both fractions when expressed per 100 mg dry weight increased greatly before the 14th day and both fractions began to decrease after this stage The 1 M sodium chloride-soluble collagen hydroxyproline of the skin formed a slight exception for the mean value continued to increase slightly between the 14th and 16th days before it began to decrease The values for free proline and free amino nitrogen likewise showed an increase or no change between the 10th and 14th days and a decrease thereafter except the free amino nitrogen of the skin, which also decreased before the 14th day A similar change in the contents of free proline and free amino nitrogen in whole embryos after the 14th day has also been found by CHVAPIL (1959) It is further noteworthy that the contents of deoxyribonucleic acids and ribonucleic acids (NOVIKOFF and POTTER 1948 REDDY LOMBARDO and CERECEDO 1952 MARRIAN HUGHES and WRBA 1956) and the contents of the polyamines spermine and spermidine (RAINA 1963) which are possibly connected in some way with nucleic acid and protein metabolism (refer RAINA 1963) show appreciable changes near the 14th day of development These observations suggest that the changes in the metabolism of collagen near the 14th day of development may be correlated with more generalized changes in the metabolism of chick embryos at this stage of development

The experiments in which ^{14}C-proline was administered indicated that the 1 M sodium chloride-soluble collagen hydroxyproline fraction had a very high turnover rate since the maximum specific activity of this fraction occurred as little as 3 h after isotope administration or possibly even earlier As discussed on page 55 the highly active peptide hydroxyproline fractions found by LEVINE (1962) and by HURYCH and CHVAPIL (1962 b) during incubation of the skin of young rats or of chick embryos *in vitro* are possibly included in the 1 M sodium chloride soluble collagen hydroxyproline fraction Such peptide fractions might contribute to the high peak observed if they are also present *in vivo* The microsomal collagen fraction studied by PROCKOP PETERKOFSKY and UDENFRIEND (1962) in chick embryos *in vivo* likewise showed a maximal specific activity as little as 2 hours after the administration of ^{14}C-proline to 9 day old embryos Thus it is evidently present in the high peak observed here

Since the 1 M sodium chloride soluble collagen fraction is heterogeneous in origin it is not surprising that the specific activity of this fraction decreased after the first high peak at a lower rate for the other subfractions possibly have a less rapid turnover However although resorption of the labelled proline from the chorioallantoic membrane seems to be very rapid some additional resorption may have taken place at a lower rate which

might account for the delay in the decrease in the specific activity of the 1 M sodium chloride soluble collagen hydroxyproline

The main purpose of the isotope experiments was to obtain some information on the role of free hydroxyproline in the metabolism of collagen in chick embryos The results of the experiments made without isotopes suggested that the free hydroxyproline originated largely from the 1 M sodium chloride soluble collagen hydroxyproline The results of the isotope experiments are in good agreement with this suggestion since during the first 12 h after the administration of labelled proline the specific activity of free hydroxyproline was higher in all embryos than that of insoluble hydroxyproline If the free hydroxyproline had been derived only from the insoluble hydroxyproline its specific activity would have been considerably lower than that of the insoluble hydroxyproline since the considerable amounts of unlabelled free hydroxyproline formed before the administration of the isotope dilute the specific activity of the labelled free hydroxyproline However it is not impossible that part of the free hydroxyproline was derived from the insoluble hydroxyproline

The results further suggest that the bulk of the free hydroxyproline of chick embryos is probably not derived from the activated hydroxyproline although such an origin for free hydroxyproline has been considered possible during the incubation of rat cartilage *in vitro* (DAUGHADAY and MARIZ 1962 b) Nor did the investigation of PROCKOP PETERKOFSKY and UDENFRIEND (1962) with chick embryos *in vivo* in which the specific activities were measured at quite short intervals during a period of 6 hours show any peak in free hydroxyproline that might suggest the derivation of free hydroxyproline from activated hydroxyproline These workers suggested that free hydroxyproline may be derived from the catabolism of one of the more highly labelled protein fractions probably of the 15 000 × *g* fraction in their fractionation system Investigations on the specific activity of the hydroxyproline in rat urine likewise indicated that hydroxyproline of urine is not derived from activated hydroxyproline Apparently a part of the hydroxyproline present in the urine of young rats is derived from soluble collagens although catabolism of insoluble collagen also occurs (LINDSTEDT and PROCKOP 1961 PROCKOP 1962 b)

It is of interest that in chick embryos the specific activity of insoluble hydroxyproline also increased rapidly and showed a maximum as little as 9 h after administration of the isotope The decrease observed thereafter was mainly due to synthesis of new unlabelled hydroxyproline but some catabolism of insoluble hydroxyproline may also have occurred

EFFECT OF CORTISONE ON HYDROXYPROLINE-CONTAINING FRACTIONS IN CHICK EMBRYOS

The purpose of this part of the investigation was to study how the cortisone induced inhibition of collagen formation is reflected in the contents of the various hydroxyproline-containing fractions expressed per unit dry weight and as a percentage of the total hydroxyproline The total amounts of hydroxyproline per embryo were also calculated in the experiments with whole embryos since the investigation of CHVAPIL (1959) suggested that when the effect of cortisone on total hydroxyproline is studied it may be more correct to express the values in this way than per unit dry weight Most experiments were made with whole embryos but finally one series was also used to investigate whether the changes observed in whole embryos were similar to those that occurred in the skin A short preliminary report of the results obtained in whole embryos has already been published (KIVIRIKKO 1963 a)

The cortisone effect was induced with cortisone acetate (ADRESON N V Organon Oss[1]) It was used as a suspension diluted with 0 9 per cent sodium chloride solution to contain the amount injected in 0 1 ml Injections were made onto the chorioallantoic membrane Control embryos received corresponding injections of 0 9 per cent sodium chloride

CONTENT OF HYDROXYPROLINE IN THE FRACTIONS OF WHOLE EMBRYOS AFTER ADMINISTRATION OF CORTISONE

EFFECT OF A SINGLE INJECTION

Effect of a single injection at the age of 10 days

The experiments dealing with the effect of cortisone were started using a single injection at the age of 10 days Two series of experiments were made designated as series A and B

[1] Kindly supplied by Mr HARRI WILLAMO *Mag Phil Organon Finland*

Table 6 *Effect of a single injection of cortisone acetate at the age of 10 days on the wet weights and relative dry weights of the embryos on different days of development*

Day and series	Group	Number of embryos	Wet weight g ± S D	Relative dry weight mg/g wet wt ± S D
10 A	Controls	3 × 2[a]	2 39 ± 0 30	73 ± 1
11 A	Controls	6	3 34 ± 0 36	83 ± 2
	100 µg	6	1 ?88 ± 0 15	*87 ± 0
	300 µg	5	*2 72 ± 0 19	*90 ± 3
1? A	Controls	5	4 16 ± 0 38	88 ± 2
	100 µg	6	4 45 ± 0 36	91 ± 3
	300 µg	6	*3 38 ± 0 31	93 ± 5
	900 µg	6	*3 18 ± 0 35	90 ± 10
14 A	Controls	6	7 40 ± 0 9?	112 ± 7
	100 µg	6	7 14 ± 0 4	10? ± 4
	300 µg	6	*4 74 ± 0 50	*102 ± 8
	900 µg	5	*3 91 ± 0 31	105 ± 4
16 B	Controls	6	14 27 ± 0.59	164 ± 6
	100 µg	6	12 03 ± 0 56	*153 ± 7
	300 µg	?	*8 82 ± 1 57	*123 ± 9
18 A	Controls	6	16 58 ± 0 84	195 ± 19
	300 µg	2	10 76 ± 0 94	163 ± 7
B	Controls	5	17 39 ± 1 25	215 ± 8
	100 µg	5	16 ?3 ± 0 6?	*196 ± 5
	300 µg	4	9 55 ± 2 03	*145 ± 17

The significance of the differences as compared with the controls is denoted by = P < 0 05 = P < 0 01 * = P < 0 001

3 pooled samples 2 embryos in each

In this group the ± deviation is given and the significance of the differences is not calculated

Dose of cortisone acetate

Weights of the embryos — The number of embryos in the different groups their wet weights and relative dry weights are presented in Table 6 All doses of cortisone used in the experiments presented in the table caused a delay in the weight gain of the embryos The effect of 30 µg of cortisone acetate was also studied but since this dose caused no significant changes in the weights of the embryos or in the contents of the various hydroxyproline-containing fractions the results are not presented Table 6 further indicates that the administration of cortisone was followed by marked changes in the water content of the treated embryos Initially after the

EFFECT OF CORTISONE ON HYDROXYPROLINE-CONTAINING FRACTIONS IN CHICK EMBRYOS

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The cortisone effect was induced with cortisone acetate (ADRESON N V Organon Oss[1]) It was used as a suspension diluted with 0 9 per cent sodium chloride solution to contain the amount injected in 0 1 ml Injections were made onto the chorioallantoic membrane Control embryos received corresponding injections of 0 9 per cent sodium chloride

CONTENT OF HYDROXYPROLINE IN THE FRACTIONS OF WHOLE EMBRYOS AFTER ADMINISTRATION OF CORTISONE

EFFECT OF A SINGLE INJECTION

Effect of a single injection at the age of 10 days

The experiments dealing with the effect of cortisone were started using a single injection at the age of 10 days Two series of experiments were made designated as series A and B

[1] Kindly supplied by Mr HARRI WILLAMO Mag Phil Organon Finland

Table 7 Effect of a single injection of cortisone acetate at the age of 10 days on th hydroxyproline-containing fractions in whole embryos on different days of development expressed as µg/100 mg dry weight of the embryo or as a percentage of the total hydroxyproline[4]

Day and series	Group[5]	Hydroxyproline					
		Free		1 M NaCl soluble collagen		Total	Total in embryo in µg
		µg/100 mg	of total	µg/100 mg	of total	µg/100 mg	
10 A	Controls	27 0	7 67	37 8	10 72	352	621
11 A	Controls	33 1	7 47	39 4	8 79	445	1260
	100 µg	[1]27 1	[3]5 40	38 7	[1]7 68	[1]504	1260
	300 µg	[3]21 6	4 80	37.3	[3]7 27	[1]516	1280
12 A	Controls	46 4	7 70	50 6	8 40	604	2530
	100 µg	37 7	5 95	50 8	8 00	640	2580
	300 µg	[3]32 1	[3]4 78	49 9	[1]7 45	675	2110
	900 µg	[3]26 7	[3]3 60	46 5	[3]6 22	752	2130
14 A	Controls	62 7	8 18	65 4	8 53	770	6640
	100 µg	[1]72 2	8 52	68 4	7 99	855	6450
	300 µg	[3]34 4	[3]3 55	70 6	[1]7 24	[3]985	[3]4810
	900 µg	[3]23 4	[3]2 64	66 8	7.31	938	[3]3830
16 B	Controls	39 1	4 24	67 2	7.34	919	21420
	100 µg	43 8	3 95	[3]73 3	6 66	[3]1103	20230
	300 µg	[3]60 2	4 81	[3]82 1	[1]6 51	[3]1264	[3]13870
18 A	Controls	18 1	2 22	61 6	7 42	915	29120
	300 µg	58 5	4 13	69.5	4 91	1415	25650
B	Controls	18 1	—	67 4	—	—	—
	100 µg	19 6	—	[3]74 4	—	—	—
	300 µg	[3]72 1	—	[1]76 4	—	—	—

The significance of the differences as compared with the *controls is denoted by* = P < 0 05 [2] = P < 0 01 [3] = P < 0 001 In 18 day old cortisone treated embryos of series A the significance of the differences is not *calculated*

[4] The values for *insoluble hydroxyproline* and the standard deviations for all values are given in Table 16 in the Appendix

[5] Dose of cortisone acetate

100 mg dry weight during the first 4 days after the *injection* However when this fraction was expressed as a percentage of the total hydroxyproline the values were significantly lower in the cortisone treated embryos than in the controls Later this fraction when *expressed per 100 mg dry*

injection the cortisone-treated embryos contained more dry matter per g wet weight than the controls and later they contained less dry matter per g wet weight than the controls

Hydroxyproline-containing fractions — The contents of free 1 M sodium chloride-soluble collagen and total hydroxyproline expressed as μg/100 mg dry weight of the embryo and as a percentage of the total hydroxyproline and the total amounts of hydroxyproline per embryo are presented in Table 7 The effect of 100 μg and 300 μg of cortisone acetate on the content of free hydroxyproline is also shown in Fig 11

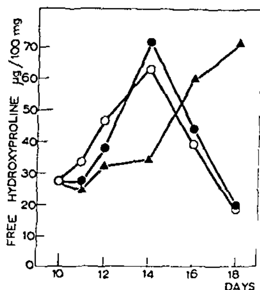


Fig 11 Effect of a single injection of cortisone acetate at the age of 10 days on the content of free hydroxyproline in whole embryos expressed as μg/100 mg dry weight of the embryo ○ Controls ● 100 μg cortisone acetate ▲ 300 μg cortisone acetate In 18 day old embryos only the values of series B are presented Abscissa Age of embryos in days

After the administration of cortisone the content of *free hydroxyproline* was initially considerably lower in the treated embryos than in the controls both when expressed as μg/100 mg dry weight and when expressed as a percentage of the total hydroxyproline This stage was later followed by a second stage in which the content of free hydroxyproline per 100 mg dry weight and as a percentage of the total hydroxyproline was higher in the treated embryos than in the controls The second stage was reached earlier in those embryos that received 100 μg of cortisone acetate than in those embryos that received 300 μg

The administration of cortisone had no significant effect on the content of *1 M sodium chloride-soluble collagen hydroxyproline* when expressed per

Table 7 Effect of a single injection of cortisone acetate at the age of 10 days on the hydroxyproline-containing fractions in whole embryos on different days of development expressed as μg/100 mg dry weight of the embryo or as a percentage of the total hydroxyproline [a]

Day and series	Group [b]	Hydroxyproline: Free, μg/100 mg	Hydroxyproline: Free, % of total	Hydroxyproline: 1 M NaCl soluble collagen, μg/100 mg	Hydroxyproline: 1 M NaCl soluble collagen, % of total	Hydroxyproline: Total, μg/100 mg	Total in embryo in μg
10 A	Controls	27 0	7 67	37 8	10 72	352	621
11 A	Controls	33 1	7 47	39 4	8 79	445	1260
	100 μg	* 27 1	5 40	38 7	* 7 68	* 504	1260
	300 μg	* 24 6	* 4 80	37 3	* 7 27	* 516	1280
12 A	Controls	46 4	7 70	50 6	8 40	604	2530
	100 μg	* 37 7	* 5 95	50 8	8 00	640	2580
	300 μg	* 32 1	* 4 78	49 9	* 7 45	675	2110
	900 μg	* 26 7	* 3 60	46 5	* 6 22	* 752	2130
14 A	Controls	62 7	8 18	65 4	8 53	770	6640
	100 μg	* 72 2	8 52	68 4	7 99	* 855	6150
	300 μg	* 34 4	* 3 55	0 6	* 7 21	98[illegible]	* 4810
	900 μg	* 23 4	* 2 64	66 8	7.34	938	* 3830
16 B	Controls	39 1	4 24	67 2	7 31	919	21420
	100 μg	43 8	3 95	* 73 3	6 66	* 1103	20230
	300 μg	60 2	4 81	* 82 1	* 6 51	* 1264	* 13870
18 A	Controls	18 1	2 92	61 6	7 42	915	29420
	300 μg	58 5	4 13	69 5	4 91	1415	25650
B	Controls	18 1	—	67 4	—	—	—
	100 μg	19 6	—	* 74 4	—	—	—
	300 μg	72 1	—	* 76 4	—	—	—

The significance of the *differences* as compared with the controls is denoted by = $P < 0.05$ = $P < 0.01$ = $P < 0.001$ In 18-day-old *cortisone treated embryos* of series A the significance of the differences is not calculated

[a] The values for *insoluble hydroxyproline* and the *standard deviations* for all values are given in Table 16 in the Appendix

[b] Dose of cortisone *acetate*

100 mg dry weight during the first 4 days after the *injection However* when this fraction was expressed as a percentage of the total hydroxyproline the values were significantly lower in the cortisone treated embryos than in the controls Later this fraction *when expressed* per 100 mg dry

weight increased above the values of the controls This increase was considerably smaller, however than that of free hydroxyproline and 8 days after the injection the content of 1 M *sodium chloride-soluble collagen* hydroxyproline was still below that of the controls expressed as a percentage of the total hydroxyproline

The contents of *insoluble* and *total hydroxyproline* were higher in the cortisone treated embryos than in the controls when expressed per 100 mg dry weight But the absolute amounts of insoluble and total hydroxyproline were unchanged or lower than in the controls When the content of insoluble hydroxyproline was expressed as a percentage of the total hydroxyproline an increase corresponding to the decrease in free and 1 M sodium chloride-soluble collagen hydroxyproline was observed

In the content of *peptide hydroxyproline no significant changes were* observed after the administration of cortisone Since the error of the present method was considerably greater in this fraction than in the other fractions (see p 32) no small changes possibly occurring in the peptide hydroxyproline would have been detected

Since the administration of cortisone caused a retardation in the weight gain of the embryos the question arose whether the cortisone treated embryos should be compared with controls of the same weight rather than with controls of the same age When such a comparison was made it was observed that qualitatively the initial changes were similar to those described above although there were quantitative differences between these two types of comparison Thus all the changes described above expressed as percentages of the total hydroxyproline appeared still greater in comparisons between embryos of the same weight The differences between the cortisone treated embryos and the controls in the contents of insoluble and total hydroxyproline expressed per 100 mg dry weight were likewise greater in this than in the previous comparison whereas the differences in the content of free hydroxyproline were smaller but nevertheless statistically significant in *some groups of embryos*

Free proline free amino nitrogen total proline and total nitrogen — The effects of 100 μg and 300 μg of cortisone acetate on the contents of free proline and free amino nitrogen expressed as μg/100 mg dry weight of the embryo are presented in Figs 12 and 13 The content of *free proline* after administration of cortisone like the content of free hydroxyproline (cf Fig 11 p 60) was initially lower in the cortisone treated embryos than in the controls (in 11 day-*old embryos after 300 μg* $P < 0\ 05$ *in 12 day old* embryos after 100 μg $P < 0\ 01$) In contrast to the free hydroxyproline

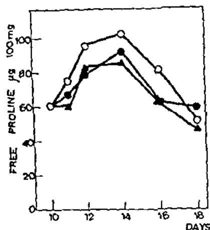


Fig 12

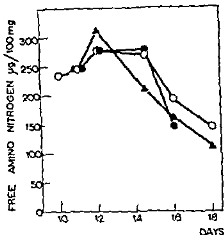


Fig 13

Fig 12 Effect of a single injection of cortisone acetate at the age of 10 days on the content of free proline in whole embryos expressed as µg/100 mg dry weight of the embryo ○ Controls ● 100 µg cortisone acetate ▲ 300 µg cortisone acetate In 18 day-old embryos only the values of series B are presented Abscissa Age of embryos in days

Fig 13 Effect of a single injection of cortisone acetate at the age of 10 days on the content of free amino nitrogen in whole embryos expressed as µg/100 mg dry weight of the embryo ○ Controls ● 100 µg cortisone acetate ▲ 300 µg cortisone acetate In 18-day-old embryos only the values of series A were determined Abscissa Age of embryos in days

however the content of free proline remained lower in the cortisone treated embryos than in the controls even during the second stage when the content of free hydroxyproline was higher than in the controls

The content of *free amino nitrogen* in the cortisone treated embryos did not differ significantly from that in the controls at the age of 11 or 12 days Thereafter the values were significantly lower in the cortisone treated embryos than in the controls (at the age of 14 days with 300 µg $P < 0.05$ at the age of 16 days with 100 µg $P < 0.01$ with 300 µg $P < 0.05$)

The administration of cortisone had no effect on the contents of *total proline* or *total nitrogen* and therefore these values are not presented in detail

Effect of a single injection at the age of 8 days

The increase in the content of free hydroxyproline observed in the 18 day old embryos of series A which had received 300 µg of cortisone acetate at the age of 10 days might have been due to a delay in reaching the maximum value observed in the controls at the age of 14 days Therefore in series B

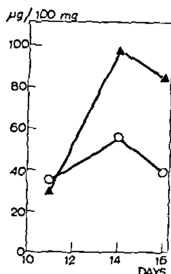


Fig 14

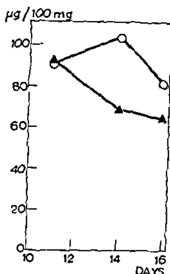


Fig 15

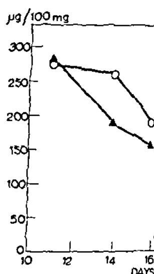


Fig 16

Figs 14 15 and 16 Effect of a single injection of cortisone acetate at the age of 8 days on the content of free hydroxyproline (Fig 14) free proline (Fig 15) and free amino nitrogen (Fig 16) in whole embryos expressed as μg/100 mg dry weight of the embryo O Controls ▲ 300 μg cortisone acetate Abscissa Age of embryos in days

the effect of a single injection of 300 μg of cortisone acetate at the age of 8 days was also investigated

The contents of free hydroxyproline free proline and free amino nitrogen in this experiment are shown in Figs 14 15 and 16 The results indicated that there was a great increase in free hydroxyproline in the cortisone-treated embryos even at the age of 14 days although the controls had a maximum value at this stage of development

In the present experiment as in the experiments in which a single injection of 300 μg of cortisone acetate was given at the age of 10 days the contents of free proline and free amino nitrogen in the cortisone treated embryos were below those in the controls during the stage when the content of free hydroxyproline was greatly increased

EFFECT OF REPEATED INJECTIONS

The foregoing experiments suggested that the true effect of cortisone would be to decrease the contents of free and 1 M sodium chloride soluble collagen hydroxyproline compared with the controls and that the later increases in the free hydroxyproline above the values of the controls are secondary Therefore the effect of repeated administration of cortisone during

a period of 8 days was studied in series C The administration of cortisone was begun at the age of 8 days and the analysis was made at 16 days

Cortisone was given in amounts depending on the weights of the control embryos which were known from the earlier experiments The following daily weights were taken as a basis for the dosage of cortisone (age in days — weight in grams) 8 — 1 0 9 — 1 5 10 — 2 5 11 — 3 3 12 — 5 13 — 7 14 — 9 and 15 — 11 The daily doses of cortisone acetate used were 3 µg/g 10 µg/g and 30 µg/g The corresponding total doses given during 8 days were thus 121 µg 403 µg and 1210 µg In addition to these groups one group received injections of 300 µg at the ages of 8 11 and 14 days and one group only one injection of 300 µg at the age of 8 days The controls received daily injections of 0 1 ml of 0 9 per cent sodium chloride

Weights of the embryos — The number of embryos in each group the weights of the embryos and their relative dry weights are presented in Table 8 Even the smallest dose of cortisone used retarded the weight gain

Table 8 Effect of repeated injections of cortisone acetate on the wet weights and relative dry weights of the embryos at the age of 16 days

Group	Number of embryos	Wet weight g ± S D	Relative dry wt. mg g wet wt
Controls	8	13 40 ± 1 51	162 ± 9
3 µg/g 8—15	6	[1] 11 84 ± 0 85	157 ± 13
10 µg/g 8—15	7	[3] 9 20 ± 1 17	[1] 15? ± 7
30 µg g 8—15	6	[3] 7 10 ± 0 73	[3] 105 ± 12
300 µg 8 11 14	5	[3] 5 83 ± 0 40	[3] 111 ± 10
300 µg 8	6	[3] 7 10 ± 1 4?	[3] 112 ± 14

The significance of the differences as compared with the controls is denoted by [1] = P < 0 05 [2] = P < 0 01 = P < 0 001

[4] Dose of cortisone acetate and age of embryos in days when the cortisone was administered

of the embryos In addition the cortisone treated embryos contained more water than the controls of the same age but especially those embryos that received daily injections of 3 µg/g or 10 µg/g of cortisone acetate contained less water than untreated embryos of the same weight usually contain

Hydroxyproline containing fractions — The contents of free 1 M sodium chloride soluble collagen and total hydroxyproline expressed as µg/100 mg dry weight of the embryo and as a percentage of the total hydroxyproline

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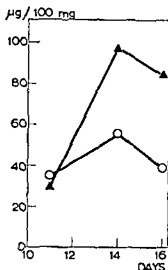


Fig 14

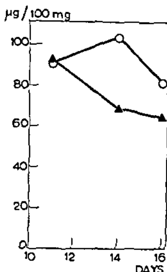


Fig 15

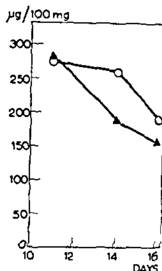


Fig 16

Figs 14 15 and 16 Effect of a single injection of cortisone acetate at the age of 8 days on the content of free hydroxyproline (Fig 14) free proline (Fig 15) and free amino nitrogen (Fig 16) in whole embryos expressed as μg/100 mg dry weight of the embryo O Controls ▲ 300 μg cortisone acetate Abscissa Age of embryos in days

the effect of a single injection of 300 μg of cortisone acetate at the age of 8 days was also investigated

The contents of free hydroxyproline free proline and free amino nitrogen in this experiment are shown in Figs 14 15 and 16 The results indicated that there was a great increase in free hydroxyproline in the cortisone treated embryos even at the age of 14 days although the controls had a maximum value at this stage of development

In the present experiment as in the experiments in which a single injection of 300 μg of cortisone acetate was given at the age of 10 days the contents of free proline and free amino nitrogen in the cortisone-treated embryos were below those in the controls during the stage when the content of free hydroxyproline was greatly increased

EFFECT OF REPEATED INJECTIONS

The foregoing experiments suggested that the true effect of cortisone would be to decrease the contents of free and 1 M sodium chloride soluble collagen hydroxyproline compared with the controls, and that the later increases in the free hydroxyproline above the values of the controls are secondary Therefore the effect of repeated administration of cortisone during

Table 10 Effect of repeated injections of cortisone acetate on the contents of free proline and free amino nitrogen in whole embryos at the age of 16 days expressed as µg/100 mg dry weight of the embryo

Group[a]	Free proline mean ± S D	Free amino-N mean ± S D
Controls	60.9 ± 12.8	200 ± 28
3 µg/g 8—15	[2]4[illegible].0 ± 3.8	[1]167 ± 22
10 µg/g 8—15	[2]43.9 ± 4.[illegible]	[2]159 ± 18
30 µg/g 8—15	38.7 ± 3.8	[2]160 ± 14
300 µg 8 11 14	3[illegible].5 ± 5.4	[1]149 ± 14
300 µg 8	[1]48.3 ± 6.5	181 ± 29

The significance of the differences as compared with the controls is denoted by [1] = $P < 0.05$ [2] = $P < 0.01$

[a] Dose of cortisone acetate and age of embryos in days when the cortisone was administered

Free proline free amino nitrogen total proline and total nitrogen — In all the cortisone treated groups the contents of free proline and free amino nitrogen were lower than in the controls (Table 10) In those groups in which the content of free hydroxyproline was below that of the controls the percentage change in the content of free proline compared with the controls was of the same magnitude as the change in free hydroxyproline except in those groups that received 10 µg/g or 30 µg/g daily In these groups the change in free hydroxyproline was greater than that in free proline The percentage change in free amino nitrogen was smaller than the change in free hydroxyproline in all groups in which the content of free hydroxyproline was lower than in the controls

The mean content of total proline expressed per 100 mg dry weight in the group receiving a daily dose of 30 µg cortisone acetate per g was 9 per cent lower than in the controls ($P < 0.05$) and in the group receiving 3 injections of 300 µg cortisone acetate 8 per cent lower ($P < 0.05$) In the other groups this value was within ± 2 per cent of the control value

The content of total nitrogen expressed in relation to the dry weight of the embryo did not differ significantly from that of the controls in any group

CONTENT OF HYDROXYPROLINE IN THE FRACTIONS OF THE SKIN AFTER ADMINISTRATION OF CORTISONE

To determine whether the changes observed in the hydroxyproline-containing fractions in whole embryos were similar to those found in tissues rich in collagen the effect of cortisone on the hydroxyproline-containing

Table 10 Effect of repeated injections of cortisone acetate on the contents of free proline and free amino nitrogen in whole embryos at the age of 16 days expressed as μg/100 mg dry weight of the embryo

Group[a]	Free proline mean ± S D	Free amino N mean ± S D
Controls	60 9 ± 1 8	200 ± 28
3 μg g 8—15	[2]42 0 ± 3 8	[1]167 ± 22
10 μg/g 8—15	[2]43 9 ± 4 2	[2]159 ± 18
30 μg/g 8 15	[2]38 7 ± 3 8	[1]160 ± 14
300 μg 8 11 14	[2]37 5 ± 5 4	[2]143 ± 14
300 μg 8	[1]48 3 ± 6 5	181 ± 29

The significance of the differences as compared with the controls is denoted by [1] = P < 0 05 [2] = P < 0 01

[a] Dose of cortisone acetate and age of embryos in days when the cortisone was administered

Free proline free amino nitrogen total proline and total nitrogen — In all the cortisone treated groups the contents of free proline and free amino nitrogen were lower than in the controls (Table 10) In those groups in which the content of free hydroxyproline was below that of the controls the percentage change in the content of free proline compared with the controls was of the same magnitude as the change in free hydroxyproline except in those groups that received 10 μg/g or 30 μg/g daily In these groups the change in free hydroxyproline was greater than that in free proline The percentage change in free amino nitrogen was smaller than the change in free hydroxyproline in all groups in which the content of free hydroxyproline was lower than in the controls

The mean content of total proline expressed per 100 mg dry weight in the group receiving a daily dose of 30 μg cortisone acetate per g was 9 *per cent* lower than in the controls (P < 0 05) and in the group receiving 3 injections of 300 μg cortisone acetate 8 per cent lower (P < 0 05) In the other groups this value was within ± 2 per cent of the control value

The content of total nitrogen expressed in relation to the dry weight of the embryo did *not differ significantly* from that of the controls in any group

CONTENT OF HYDROXYPROLINE IN THE FRACTIONS OF THE SKIN AFTER ADMINISTRATION OF CORTISONE

To determine whether the changes observed in the hydroxyproline containing fractions in whole embryos were similar to those found in tissues rich in collagen the effect of cortisone on the hydroxyproline-containing

fractions of the skin was studied One group of embryos received 3 μg cortisone acetate per g body weight daily between days 8 and 15 and another group 300 μg of cortisone acetate as a single injection at the age of 15 days Controls received daily injections of 0 1 ml of 0 9 per cent sodium chloride between days 8 and 15

The preparation of the skin samples was begun 8 hours before the age of 16 days in the following order first pooled sample of the embryos that received 3 μg/g daily first pooled sample of controls first pooled sample of the embryos that received 300 μg at the age of 15 days second pooled sample of the embryos that received 3 μg/g daily and so forth The preparation of this series containing three pooled samples in each group took over 13 hours Thus the preparation of the third pooled sample was begun in each group about 9 hours after the first sample of the group

Weights of the embryos — The number of embryos in each pooled sample was 4—6 The mean weights of the embryos in each group and the mean relative dry weights of the pooled skin samples and their range are given in Table 11 In both cortisone treated groups the mean weights of the embryos were below those of the controls but the difference between the group that received 300 μg of cortisone acetate and the controls was very small

Table 11 Effect of cortisone acetate on the weights of the embryos and on the relative dry weights of the pooled skin samples of 16 day old embryos in the experiments with the skin [1]

Group [3]	Number of embryos	Wt of embryos g	Relative dry wt of the pooled skin sample [2]
Controls	14	14 69	128 (116—144)
3 μg/g 8—15	15	11 67	121 (113—132)
300 μg 15	16	14 06	135 (129—141)

[1] *Three pooled samples in each group skin of 4—6 embryos in each pooled sample*
[2] mg/g wet wt mean and range
[3] Dose of cortisone acetate and age of embryos in days when it was administered

Hydroxyproline-containing fractions — The contents of free peptide 1 M sodium chloride soluble collagen insoluble and total hydroxyproline in each sample expressed both as μg/100 mg dry weight of the skin and as a percentage of the total hydroxyproline are given in Table 12 Both modes of administration of cortisone resulted in changes similar to those observed in whole embryos in the following respects The contents of free and 1 M sodium chloride-soluble collagen hydroxyproline were lower than in the controls both when expressed as μg/100 mg dry weight of the skin and when expressed as a percentage of the total hydroxyproline and the

Table 1[?] Effect of cortisone acetate on hydroxyproline-containing fractions of the pooled skin samples of 18-day-old embryos expressed as μg/100 mg dry weight of the skin and as a percentage of the total hydroxyproline

Group[2]	No[1]	Hydroxyproline								
		Free		Peptide		1 M NaCl soluble collagen		Insoluble		Total
		μg/100 mg	% of total	μg/100 mg	% of total	μg/100 mg	% of total	μg/100 mg	% of total	μg/100 mg
Controls	1	36 9	1 81	4 8	0 24	134	6 57	1860	91 4	2040
	2	34 5	1 73	2 9	0 15	131	6 58	1870	91 5	1990
	3	30 0	1 61	6 4	0 34	125	6 72	1700	91.3	1860
	Mean	33 8	1 72	4 7	0 24	130	6 62	1790	91 4	1960
3 μg/g 8–15	1	23 0	1 46	2 1	0 13	71 6	4 53	1480	93 9	1580
	2	26 7	1 55	4 1	0 24	7 8	4 57	1610	93 7	1720
	3	22 9	1 49	4 1	0 27	2 3	4 70	1440	93 6	1540
	Mean	24 2	1 50	3 4	0 21	73 9	4 58	1510	93 7	1610
300 μg 15	1	20 4	1 19	2 8	0 16	73 7	4 31	1610	94 3	1710
	2	14 2	0 82	2 4	0 14	66 3	3 84	1650	95 2	1730
	3	12 6	0 72	3 3	0 19	65 9	3 76	1670	95 3	1750
	Mean	15 7	0 91	2.8	0 16	68 6	3 97	1640	94 9	1730

No of pooled sample skin of 4–6 embryos in each

Dose of cortisone acetate and days on which it was administered

content of insoluble hydroxyproline was higher than in the controls when expressed as a percentage of the total hydroxyproline The peptide hydroxyproline did not show any changes that could be regarded as significant although the mean values for this fraction were lower than in the controls after the administration of cortisone whether expressed as μg/100 mg dry weight or as a percentage of the total hydroxyproline In contrast to the changes observed in whole embryos the contents of insoluble and total hydroxyproline in the skin were lower in the cortisone treated embryos than in the controls when expressed as μg/100 mg dry weight of the skin

The content of citrate-soluble collagen hydroxyproline decreased from the value of 3 3 (3 0–3 5) μg/100 mg dry weight of the skin in the controls to the value of 1 4 (0 9–1 8) μg/100 mg in the group that received 3 μg/g daily and to the value of 1 6 (1 0–2 2) μg/100 mg in the group that received 300 μg as a single injection

fractions of the skin was studied One group of embryos received 3 μg cortisone acetate per g body weight daily between days 8 and 15 and another group 300 μg of cortisone acetate as a single injection at the age of 15 days Controls received daily injections of 0 1 ml of 0 9 per cent sodium chloride between days 8 and 15

The preparation of the skin samples was begun 8 hours before the age of 16 days in the following order first pooled sample of the embryos that received 3 μg/g daily first pooled sample of controls first pooled sample of the embryos that received 300 μg at the age of 15 days second pooled sample of the embryos that received 3 μg/g daily and so forth The preparation of this series containing three pooled samples in each group took over 13 hours Thus the preparation of the third pooled sample was begun in each group about 9 hours after the first sample of the group

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Table 11 Effect of cortisone acetate on the weights of the embryos and on the relative dry weights of the pooled skin samples of 16 day old embryos in the experiments with the skin[1]

Group[3]	Number of embryos	Wt of embryos g	Relative dry wt of the pooled skin sample[2]
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3 μg/g 8—15	15	11 C7	121 (113—132)
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[1] Three pooled samples in each group skin of 4—C embryos in each pooled sample
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[3] Dose of cortisone acetate and age of embryos in days when it was administered

Hydroxyproline containing fractions — The contents of free peptide 1 M sodium chloride soluble collagen insoluble and total hydroxyproline in each sample expressed both as μg/100 mg dry weight of the skin and as a percentage of the total hydroxyproline are given in Table 12 Both modes of administration of cortisone resulted in changes similar to those observed in whole embryos in the following respects The contents of free and 1 M sodium chloride-soluble collagen hydroxyproline were lower than in the controls both when expressed as μg/100 mg dry weight of the skin and when expressed as a percentage of the total hydroxyproline and the

Table 1 Effect of cortisone ac tate on hydroxyproline-containing fractions of the pooled skin samples of 16-day old embryos expressed as μg/100 mg dry weight of the skin and as a percentage of the total hydroxyproline

Group [a]	No [1]	Hydroxyproline								
		Free		Peptide		1 M NaCl soluble collagen		Insoluble		Total
		μg/100 mg	of total	μg/100 mg	of total	μg/100 mg	% of total	μg/100 mg	of total	μg/100 mg
Controls	1	36 9	1 81	4 8	0 24	134	6 57	1860	91 4	2040
	2	31 5	1 73	2 9	0 15	131	6 58	1870	91 5	1990
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	Mean	33 8	1 72	4 7	0 24	130	6 62	1 90	91 4	1960
3 μg/g 8 1	1	23 0	1 46	2 1	0 13	71 6	4 53	1480	93 9	1580
	2	26 7	1 55	4 1	0 24	77 8	4 57	1610	93 7	1720
	3	22 9	1 49	4 1	0 27	72 3	4 70	1440	93 6	1540
	Mean	24 2	1 50	3 4	0 21	3 9	4 58	1510	93 7	1610
300 μg 15	1	0 4	1 19	2 8	0 16	73 7	4 31	1610	94 3	1710
	2	14 2	0 87	2 4	0 14	66 3	3 84	1650	95 2	1730
	3	12 6	0 72	3 3	0 19	6 9	3 76	16 0	95 3	1750
	Mean	15 7	0 91	2 8	0 16	68 6	3 97	1640	94 9	1730

No of pooled sample skin of 4—6 embryos in each

[a] Dose of cortisone acetate and days on which it was administered

content of insoluble hydroxyproline was higher than in the controls when expressed as a percentage of the total hydroxyproline The peptide hydroxyproline did not show any changes that could be regarded as significant although the mean values for this fraction were lower than in the controls after the administration of cortisone whether expressed as μg/100 mg dry weight or as a percentage of the total hydroxyproline In contrast to the changes observed in whole embryos the contents of insoluble and total hydroxyproline in the skin were lower in the cortisone treated embryos than in the controls when expressed as μg/100 mg dry weight of the skin

The content of citrate soluble collagen hydroxyproline decreased from the value of 3 3 (3 0—3 5) μg/100 mg dry weight of the skin in the controls to the value of 1 4 (0 9—1 8) μg/100 mg in the group that received 3 μg/g daily and to the value of 1 6 (1 0—2 2) μg/100 mg in the group that received 300 μg as a single injection

Table 13 Effect of cortisone acetate on free proline free amino nitrogen total proline and total nitrogen of the skin of 16 day old embryos expressed per 100 mg dry weight of the skin

Group [1]	Free proline µg/100 mg	Free amino N µg/100 mg	Total proline µg/100 mg	Total N mg/100 mg
Controls	69 4	189	4370	10 7
3 µg/g 8—15	55 8	157	4010	10 4
300 µg 15	67 3	167	4200	10 3

[1] Dose of cortisone acetate and days on which it was administered

Free proline free amino nitrogen total proline and total nitrogen — Although these values were slightly lower in the cortisone-treated embryos than in the controls the changes in all cases were smaller than the corresponding changes in hydroxyproline (Table 13)

DISCUSSION

Cortisone at least when administered in large doses is capable of inhibiting the formation of new collagen (for ref see pp 13—15) In the foregoing experiments the effect of this inhibition on the various hydroxyproline-containing fractions was studied in whole chick embryos and in the skin of the embryos Since all doses of cortisone used in the present study retarded the weight gain of the embryos it seems evident that the changes observed concern the pharmacological action rather than the physiological action of cortisone on collagen formation It is noteworthy however that a dose of 3 µg cortisone acetate daily per g wet weight of the embryo although it resulted in changes qualitatively similar to those produced by larger doses caused only a slight inhibition in the weight gain especially in the experiments with whole embryos

The results obtained in the present study were complicated by the fact that in the experiments with whole embryos the contents of total and insoluble hydroxyproline when expressed per unit dry weight of the embryo were higher in the cortisone treated embryos than in the controls A similar change has also been found by CHVAPIL (1959) in the content of total hydroxyproline after the administration of 1 mg of cortisone acetate to 8-day old chick embryos However he called attention to the fact that the absolute amount of hydroxyproline was lower in the cortisone-treated embryos than in the controls although its concentration was higher He therefore suggested that the synthesis of some other substances was inhibited

even more than the synthesis of collagen which would result in an increased concentration of collagen

The values calculated in the present study for the amounts of total and insoluble hydroxyproline per embryo were likewise lower than in the controls or not changed by the administration of cortisone In addition in the skin of the embryos even the contents of total and insoluble hydroxyproline expressed per unit dry weight of the skin were lower than in the controls after the administration of cortisone although the other effects of cortisone were similar to those in whole embryos Therefore it seems probable that the above described suggestion of CHVAPIL (1959) is correct and that the changes observed in the present study may be discussed in the light of inhibited collagen formation

The earlier investigations concerning the effect of cortisone on the content of free hydroxyproline in the embryos indicated that the content of free hydroxyproline was greatly increased in chick embryos 6 to 10 days after administration of 1 mg of cortisone acetate to 8-day-old embryos (ROBERTS KARNOFSKY and FRANKEL 1951 CHVAPIL 1959) but decreased in newborn rats after the administration of hydrocortisone to pregnant rats or directly to the newborn young (CHVAPIL 1958) The present study indicates that the reason for this discrepancy was that the time factor was not taken into consideration in the earlier investigations with chick embryos After administration of cortisone as a single dose or repeatedly during a period of eight days the content of free hydroxyproline was initially lower in the cortisone treated embryos than in the controls both when expressed per unit dry weight of the embryo or of the skin and when expressed as a percentage of the total hydroxyproline When cortisone was administered as a single injection this first stage was followed by a second stage during which the content of free hydroxyproline was higher in the cortisone-treated embryos than in the controls both when expressed per unit dry weight and when expressed as a percentage of the total hydroxyproline The second stage was reached earlier in those embryos that received a smaller dose of cortisone than in those that received a larger dose

From the present results it seems likely that the true effect of cortisone was to decrease the content of free hydroxyproline compared with the controls and that the increase observed later after a single injection was a secondary one It is possible that the secondary effect was due to qualitative changes in the synthesis or stability of collagen after the massive dose of exogenous cortisone was metabolized Since large doses of cortisone reduce the secretory activity of the adrenal cortex it is possible that adrenocortical hypofunction contributed to the secondary effect observed The present

results indicate that considerable danger attends the interpretation of data obtained in studies in which a substance is administered to chick embryos as a single injection and only one measurement is made some days later since secondary effects may be observed and taken for primary ones

The changes in the 1 M sodium chloride-soluble collagen hydroxyproline were on the whole similar to the changes in the free hydroxyproline The content of this fraction was initially lower in the treated embryos than in the controls both after the administration of cortisone as a single injection and after repeated administration of cortisone during eight days The change in free hydroxyproline was as a rule greater than that in the 1 M sodium chloride soluble collagen hydroxyproline and this was especially the case after administration of cortisone as a single massive dose Earlier investigations indicated a decrease in the contents of alkali soluble and neutral salt soluble collagens in the skin of guinea-pigs or rats after the administration of hydrocortisone or cortisone (SIUKO SAVELA and KULONEN 1959 SAKATA 1960 a GUNTHER and CARSTEN 1961, SETHI RAMEY and HOUCK 1961) Thus the effect of cortisone on the content of 1 M sodium chloride-soluble collagen hydroxyproline observed in the present study in whole embryos and in the skin was similar to that described earlier in more mature connective tissue

Earlier investigations concerning the effect of hydrocortisone or cortisone on the content of citrate soluble collagen in the skin of guinea pigs or rats have yielded conflicting results for both increased values (SIUKO SAVELA and KULONEN 1959 SETHI RAMEY and HOUCK 1961) and decreased values (SAKATA 1960 a GUNTHER and CARSTEN 1961 SMITH 1962 a) have been reported In the investigation of SAKATA (1960 a) in which the citrate-soluble collagen was found to have decreased the decrease in the other *fractions was even greater and thus there was a relative increase in citrate-*soluble collagen Evidently one reason for the conflicting results in the literature concerning the content of citrate-soluble collagen is that different fractions are termed citrate-soluble collagen by different investigators because the preliminary extraction of alkali soluble or neutral salt soluble collagen has been carried out with solutions of different ionic strengths In addition there are differences due to the method of homogenization of the skin samples, to the ages of the animals and to the further purification of the fractions termed citrate soluble collagen The content of citrate-soluble collagen hydroxyproline was very low in chick embryos Therefore it cannot be established whether the decrease observed in the present study after the administration of cortisone really occurred in the citrate soluble collagen hydroxyproline or whether it was due to contamination of this fraction with

small amounts of hydroxyproline from the 1 M sodium chloride soluble fraction Nevertheless it is apparent that there was no increase in the citrate-soluble collagen hydroxyproline in the present investigation

The content of peptide hydroxyproline showed no significant changes either in whole embryos or in the skin after the administration of cortisone The analytical error was considerably greater in this fraction than in the other fractions and small changes would thus not have been detected At all events it seems evident that no increase occurred in this fraction

Different opinions have been voiced in the literature as to the mode of the inhibitory action of cortisone on collagen formation CHVAPIL (1958 1959) suggested that in rat and chick embryos cortisone inhibited hydroxyproline synthesis possibly during the hydroxylation of proline Nevertheless when rat cartilage was incubated *in vitro* with ^{14}C-proline neither the amount nor the labelling of free hydroxyproline in the medium was inhibited by cortisone although the incorporation of ^{14}C proline into cartilage bound hydroxyproline was significantly reduced (DALGHADAY and MARITZ 1962 b) The finding that the contents of alkali soluble and neutral salt-soluble collagens decreased in the skin of guinea pigs and rats after administration of hydrocortisone or cortisone (SIUKO SAVELA and KULONEN 1959 SAKATA 1960 a SETHI RAMEY and HOUCK 1961) suggests inhibition of the formation of collagen before the synthesis of soluble collagens In agreement with this the experiments of MAZUROV and OREHOVIĆ (1960) with ^{14}C glycine indicated that the synthesis of citrate soluble procollagen was greatly reduced after the administration of cortisone SAKATA (1960 a) regarded it as possible that cortisone not only suppressed the synthesis of collagen but also promoted its breakdown since the content of citrate soluble collagen expressed as a percentage of total collagen was increased in his experiments GUNTHER and CARSTEN (1961) who found that the contents of soluble collagens were increased after adrenalectomy suggested that this effect was possibly due to altered stability of the collagen fibrils caused by changes in the mucopolysaccharides of the ground substance of the connective tissue

The changes observed in the present investigation suggest an inhibition of collagen formation at an early stage by cortisone Such an inhibition would cause a decreased content of soluble collagens and also of free hydroxyproline if the latter is derived largely from the 1 M sodium chloride-soluble collagen hydroxyproline as suggested earlier in this study It is possible that an altered stability of collagen fibres caused by cortisone contributed to the changes observed It seems unlikely that cortisone also increased the catabolism of collagen primarily for the content of free hydroxyproline was

results indicate that considerable danger attends the interpretation of data obtained in studies in which a substance is administered to chick embryos as a single injection and only one measurement is made some days later since secondary effects may be observed and taken for primary ones

The changes in the 1 M sodium chloride-soluble collagen hydroxyproline were on the whole similar to the changes in the free hydroxyproline The content of this fraction was initially lower in the treated embryos than in the controls both after the administration of cortisone as a single injection and after repeated administration of cortisone during eight days The change in free hydroxyproline was as a rule greater than that in the 1 M sodium chloride-soluble collagen hydroxyproline and this was especially the case after administration of cortisone as a single massive dose Earlier investigations indicated a decrease in the contents of alkali soluble and neutral salt-soluble collagens in the skin of guinea pigs or rats after the administration of hydrocortisone or cortisone (SIUKO SAVELA and KULONEN 1959 SAKATA 1960 a GUNTHER and CARSTEN 1961 SETHI RAMEY and HOUCK 1961) Thus the effect of cortisone on the content of 1 M sodium chloride-soluble collagen hydroxyproline observed in the present study in whole embryos and in the skin was similar to that described earlier in more mature connective tissue

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SUMMARY

The purpose of the present investigation was to make a simultaneous study of the low molecular and macromolecular hydroxyproline-containing fractions during the formation of collagen in developing chick embryos. In addition the effect of administration of cortisone on the contents of these fractions was studied.

A procedure suitable for the isolation of the various hydroxyproline containing fractions from the same tissue sample was worked out. The following fractions were studied 1) free hydroxyproline 2) peptide hydroxyproline soluble in 80 per cent ethanol 3) 1 M sodium chloride soluble collagen hydroxyproline insoluble in 80 per cent ethanol 4) citrate soluble collagen hydroxyproline insoluble in 1 M sodium chloride 5) insoluble hydroxyproline 6) total hydroxyproline. By the methods used for the determination of hydroxyproline optimum recoveries and specific colour reactions were obtained in all the fractions in which they were used.

The experiments carried out to test the reliability of the fractionation procedure showed that neither losses of hydroxyproline nor artificial formation of free hydroxyproline occurred during the procedure. In addition the amount of free hydroxyproline extracted was independent of the ionic strength of the sodium chloride solution used.

In the first part of the investigation the contents of the various fractions were determined in normal whole embryos between the ages of 2½ and 20 days and in the skin of embryos between the ages of 12 and 20 days. The values for hydroxyproline in the various fractions were expressed per 100 mg dry weight of the embryo or of the skin and as a percentage of the total hydroxyproline. In addition in this part experiments are reported in which the specific activities of the hydroxyproline in some of the fractions were studied after the administration of ^{14}C proline.

All the fractions investigated except citrate-soluble collagen hydroxyproline were present in the embryo after an incubation period of as little as 2½ days. At this stage of development the content of total hydroxy

decreased It is not possible to establish whether the inhibition was due to a direct action of cortisone on the fibroblasts or whether it was caused indirectly by a decrease in the amount of amino acids available for the synthesis of collagen The latter alternative cannot be excluded because there was often some decrease in the free proline and free amino nitrogen observed after the administration of cortisone Neither is it possible to decide whether the effects of large doses of cortisone on the secretory activity of other endocrine glands contributed to the changes observed

Both in this part of the investigation and in the preceding part the changes in the hydroxyproline containing fractions were similar in whole embryos and in the skin with but a few slight exceptions Thus it seems probable that whole embryos can be used for investigations on the formation of collagen in chick embryos The fact that after administration of cortisone the content of total hydroxyproline was higher in whole embryos than in the controls but lower in the skin of the embryos than in the controls when expressed per unit dry weight indicates that there may be difficulty in selecting a basis for the expression of the changes observed This difficulty may be partly avoided by expressing the fractions as percentages of the total hydroxyproline The use of whole embryos is preferable to the use of the skin, since the preparation of the skin of the embryos is troublesome and time consuming

In the last part of the investigation the effect of cortisone on the various hydroxyproline-containing fractions was studied in whole embryos and in the skin of the embryos

After the administration of cortisone as a single injection or repeatedly during a period of eight days the contents of free and 1 M sodium chloride-soluble collagen hydroxyproline were lower in the treated embryos than in the controls both when expressed per 100 mg dry weight of the embryo or of the skin and when expressed as a percentage of the total hydroxyproline When the cortisone was administered as a single injection a second stage followed During this the contents of free and 1 M sodium chloride-soluble collagen hydroxyproline expressed per 100 mg dry weight and the content of free hydroxyproline expressed as a percentage of the total hydroxyproline were higher in the cortisone-treated embryos than in the controls

The contents of peptide and citrate soluble collagen hydroxyproline did not show appreciable changes after the administration of cortisone

The contents of insoluble and total hydroxyproline in whole embryos were higher after the administration of cortisone than in the controls when expressed per 100 mg dry weight but their absolute amounts per embryo were lower than in the controls or unchanged In the skin of the embryos the contents of insoluble and total hydroxyproline were lower in the cortisone-treated embryos than in the controls even when expressed per 100 mg dry weight of the skin The relative content of insoluble hydroxyproline expressed as a percentage of the total hydroxyproline was higher in the cortisone treated embryos than in the controls

To study the specificity of the changes in hydroxyproline of normal and cortisone treated embryos the contents of free proline free amino nitrogen total proline and total nitrogen were determined in the same embryos as the values for hydroxyproline

In the discussion which is divided into two parts one appearing after each of the two principal parts of the investigation special attention was paid to the significance of the various hydroxyproline-containing fractions in the metabolism of collagen during embryonic development in chicks

proline was 39.5 μg/100 mg dry weight, whereas before incubation in hydrolysed egg yolk it was considerably less than 2 μg/100 mg and in egg white considerably less than 5 μg/100 mg dry weight.

The content of total hydroxyproline per 100 mg dry weight increased continuously during embryonic development. The increase was about 27-fold between the ages of 2½ and 20 days in whole embryos and about 10-fold between the ages of 12 and 20 days in the skin. In the case of insoluble hydroxyproline the corresponding values were still greater.

The relative content of insoluble hydroxyproline was 51.5 per cent of the total hydroxyproline in 2½-day-old embryos and had increased to the value of 92.1 per cent in 20-day-old embryos. In the skin of the embryo the corresponding values were 67.4 per cent in 12-day old embryos and 96.1 per cent in 20-day old embryos.

The contents of free and 1 M sodium chloride-soluble collagen hydroxyproline showed a maximum value at the age of 14 days when expressed per 100 mg dry weight, except the 1 M sodium chloride soluble collagen hydroxyproline in the skin, the maximum value of which was reached at the age of 16 days. After the maximum a rapid decrease occurred in the content of free hydroxyproline, whereas the content of 1 M sodium chloride soluble collagen hydroxyproline decreased only slightly. The values for free hydroxyproline were 6.8 μg/100 mg in 2½ day old, 62.3 μg/100 mg in 14 day old and 16.4 μg/100 mg in 20-day-old embryos. Even the absolute amount of free hydroxyproline in the embryo decreased after the 16th day of development. Free and 1 M sodium chloride-soluble collagen hydroxyproline formed a considerable part of the total hydroxyproline during the early stages of development and a marked decrease in the relative contents of these fractions occurred during development.

Throughout embryonic development the contents of peptide hydroxyproline and citrate soluble collagen hydroxyproline were very low both in whole embryos and in the skin.

When ^{14}C-proline was administered to 11-day-old embryos, the specific activity of 1 M sodium chloride-soluble collagen hydroxyproline showed a maximum value as little as 3 hours after isotope administration. During the first 12 hours the specific activity of free hydroxyproline was considerably lower than that of 1 M sodium chloride-soluble collagen hydroxyproline but higher than that of insoluble hydroxyproline. The specific activities of all the fractions decreased towards the end of the 30-hour observation period. However, the decrease in the specific activity of insoluble hydroxyproline was so small that it was mainly due to the synthesis of new unlabelled hydroxyproline.

In the last part of the investigation the effect of cortisone on the various hydroxyproline-containing fractions was studied in whole embryos and in the skin of the embryos

After the administration of cortisone as a single injection or repeatedly during a period of eight days the contents of free and 1 M sodium chloride soluble collagen hydroxyproline were lower in the treated embryos than in the controls both when expressed per 100 mg dry weight of the embryo or of the skin and when expressed as a percentage of the total hydroxyproline When the cortisone was administered as a single injection a second stage followed During this the contents of free and 1 M sodium chloride-soluble collagen hydroxyproline expressed per 100 mg dry weight and the content of free hydroxyproline expressed as a percentage of the total hydroxyproline were higher in the cortisone treated embryos than in the controls

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APPENDIX

Table 14 Hydroxyproline containing fractions in the samples of skin of normal embryos

Age in days	S	No	Dry wt of sample	Number of embryos	Mean wt of embryos	Hydroxyproline [a]					
						Free	Pept	1 M NaCl soluble collagen	Citrate soluble collagen	In soluble	Total
12	L	1	9	12	5 08	42 3	3 3	50 6	1 0	191	28[illegible]
		2	86	10	5 37	42 8	2 0	47 9	1 5	200	293
14	I	1	129	10	10 90	50 1	7 7	101	—	981	1140
	I	1	97	7	9 49	52 2	4 3	119	3 7	864	1040
16	K	1	155	4	16 69	20 5	2 7	123	5 0	1770	1930
		2	169	5	15 83	20 2	—	103	4 0	1720	1850
	I	1	116	4	14 42	36 9	4 8	134	3 0	1860	2040
		2	144	5	14 98	34 5	2 9	131	3 5	1820	1990
		3	124	5	14 67	30 0	6 4	127	3 3	1700	1860
20	K	1	161	4	28 90	15 2	8 1	93 1	4 3	2730	2850
			162	3	28 30	12 7	9 9	90 1	4 1	2830	2940

Series mg/g wet weight g μg/100 mg dry weight of the skin

Received 0 1 ml of 0 9 per cent sodium chloride daily between days 8 and 15

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APPENDIX

Table 14 Hydroxyproline-containing fractions in the samples of skin of normal embryos

Age in days	S[1]	No	Dry wt of sample[2]	Number of embryos	Mean wt of embryos	Hydroxyproline[3]					
						Free	Pept	1 M NaCl soluble collagen	Citrate soluble collagen	Insoluble	Total
12	L	1	9	12	5 08	42 3	3 3	50 6	1 0	191	297
		2	8(	10	5 37	42 8	2 0	47 9	1 5	200	293
14	K	1	129	10	10 90	50 1	7 7	101		981	1140
	L	1	9/	7	9 49	52 2	4 3	119	3 7	864	1040
16	K	1	155	4	16 69	26 5	2 7	129	5 0	[illegible]	1930
		2	169	5	15 83	26 2	—	103	4 0	1720	1850
	L	1	116	4	14 42	36 9	4 8	134	3 0	1860	2010
		2	144	5	14 98	34 5	2 9	131	3 5	1820	1990
		3	124	5	14 (	30 0	6 4	12,	3 3	1700	1860
20	K	1	161	4	28 90	15 2	8 1	93 1	4 3	2730	2850
		2	162	3	28 36	12 7	9 9	90 1	4 1	2830	2940

Series mg g wet wei ht [3] g μg/100 mg dry weight of the skin

R ceived 0 1 ml of 0 9 per cent sodium chloride daily between days 8 and 15

Table 10. Specific activities of free, 1 M sodium chloride soluble collagen and insoluble hydroxyproline after administration of 2 μC of ^{14}C-proline to 11-day-old chick embryos

Hours after inject	Series	No	Weight g	Specific activity of hydroxyproline[1]		
				Free	1 M NaCl soluble collagen	Insoluble
3	M	1	3.80	8290	69500	5550
		2	3.98	7580	66500	6140
		3	3.89	7570	69500	5760
6	M	1	3.83	13600	33600	10200
		2	4.13	12600	28100	9700
		3	4.46	18000	41700	13100
	N	1	4.42	17100	32400	13300
		2	4.57	13100	16500	8450
9	M	1	4.05	17100	27400	11300
		2	3.96	13500	24300	11400
		3	3.94	15200	31500	13600
12	M	1	3.78	14500	22600	11300
		2	4.61	12900	17700	11400
		3	4.06	14700	23600	11800
15	M	1	4.71	11000	18600	11300
20	N	1	5.52	8130	8190	8480
		2	4.9[illegible]	8050	8980	7280
		3	5.11	7750	7720	7730
30	N	1	6.81	5100	5150	5430
		2	6.03	5250	4700	5450
		3	5.37	6100	9030	7650

[1] cpm/μmole

Table 16 Effect of a single injection of cortisone acetate at the age of 10 d tys on the hy iroxy proline-cont uning fractions in whole embryos on different days of development expresse l as µg 100 mg dry weight of the embryo or as a percentage of the total hydroxyproline

Day and series	Group	Hydroxyproline							Total in embryo in µg
		Free		1 M NaCl soluble collagen		Insoluble		Total	
		µg/100 mg	of total	µg/100 mg	of total	µg/100 mg	of total	µg 100 mg	
10 A	Contr	?7 0	7 67	37 8	10 ?2	283	80 4	3?2	621
11 A	Contr	33 1	7 47	39 4	8 ?9	373	83 ?	445	1260
		3	0 56	3 1	0 57	36	0 9	40	2 0
	100 µg	*27 1	5 40	38 7	*7 68	*439	*86 9	*501	1 ?0
		4 1	0 95	1 6	0 50	38	1 *	37	140
	300 µg	*24 6	4 80	37 3	*7 27	*454	*87 9	*516	1280
		2 1	0 54	4 0	0 58	37	1 4	37	190
12 A	Contr	46 4	? 70	50 6	8 40	507	83 9	604	2530
		6 3	1 16	1 8	0 4	23	1 2	1	310
	100 µg	3 ?	*5 95	50 8	8 00	552	86 0	640	2580
		4 9	1 11	4 4	1 4	64	2 3	60	330
	300 µg	*3? 1	*4 78	49 9	*7 45	593	*87 7	675	2110
		5 2	1 01	1 7	0 84	77	2 1	7	310
	900 µg	*26 7	3 ?0	46 5	*6 22	*678	*90 2	*?52	2130
		3 1	0 55	7 5	0 98	94	1 0	98	290
14 A	Contr	62	8 18	?5 4	8 53	637	82 7	? 0	??40
		4 4	0 86	5	0 93	5	1 9	47	1050
	100 µg	72 2	8 5?	68 4	7 99	*712	82 6	855	6450
		9 3	1 45	4 6	0 64	48	1 3	41	910
	300 µg	34 4	*3 55	0 6	7 24	*8?3	*88 5	*985	*4810
		4 9	0 74	3 6	0 91	122	1 8	119	630
	900 µg	?3 4	*2 64	66 8	34	*844	*89 7	939	*3830
		2 6	0 84	4 9	1 28	190	2 0	193	1010
16 B	Contr	39 1	4 24	67 2	7 34	812	88 4	919	21420
		5 9	0 44	3 8	0 67	45	0 4	49	1290
	100 µ	43 8	3 95	73 3	6 66	*986	*89 4	*1103	?0230
		8 1	0 45	4 7	0 56	69	0 7	77	1700
	300 µg	*60 2	4 81	82 1	*6 51	*11?1	89 ?	1264	*13870
		13 2	1 21	7 1	0 5	103	1 5	101	3990
18 A	Contr	18 1	?22	61 6	? 42	831	89 9	915	29420
		2 4	0 64	7 2	1	362	2 9	370	11910
	300 µg	58 5	4 13	69 5	4 91	1280	90 5	1415	25650
		3	0 21	0 7	0 06	4	0 1	5	3 50
B	Contr	18 1	—	67 4	—	—	—	—	—
		3 0		2 5					
	100 µg	19 6	—	74 4	—	—	—	—	—
		1 4		3 8					
	300 µg	2 1	—	6 4	—	—	—	—	—
		9 6		6 5					

The signif cance of the differences as compared with the controls is denoted by = 1 < 0 0 = P < 0 01 = P < 0 001

The values are given as mean ± S D except in 18 day-old cortisone treated embryos of s ries A wher th ± deviation is given and the significance of the differences is not calculated

In 12-day-ol I embryos p ptide hydroxyproline was not taken into consideration when th v lues for insoluble hydroxyproline were calculated since ... all embryos

Table 15 Specific activities of free 1 M sodium chloride soluble collagen and insoluble hydroxyproline after administration of ~ μC of ^{14}C proline to 11 day old chick embryos

Hours after inject	Series	No	Weight g	Specific activity of hydroxyproline[1]		
				Free	1 M NaCl soluble collagen	Insoluble
3	M	1	3 80	8290	69500	5550
		2	3 98	7580	66500	6140
		3	3 89	7570	69500	5760
6	M	1	3 89	13600	33800	10200
		2	4 13	12600	28100	9700
		3	3 46	18000	41700	13100
	N	1	3 42	17100	32400	13300
		2	4 57	13100	16500	8450
9	M	1	4 05	17100	27400	11300
		2	3 96	13500	24300	11400
		3	3 94	15200	31500	13000
12	M	1	3 78	14500	22600	11300
		2	4 61	12900	17700	11400
		3	4 06	14700	23600	11800
15	M	1	4 71	11000	18600	11300
20	N	1	5 52	8430	8190	8180
		2	4 99	8050	8980	7280
		3	5 11	7750	7720	7730
30	N	1	6 84	5400	5150	5130
		2	6 03	5250	4700	5150
		3	5 37	6100	9030	7050

[1] cpm/μmole

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HISTAMINE METABOLISM IN ADRENALECTOMIZED RATS

BY

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CONTENTS

CHAPTER I

INTRODUCTION

A Previous work on the relationship between histamine and the endocrine glands

The possible relationship between the function of histamine and the endocrine glands has been the subject of several studies This is particularly so for the adrenal glands the removal of which leads to conspicuous changes in the actions and the metabolism of histamine

Thus DALE (1920) found that removal of the adrenal glands in the cat *caused an increased sensitivity to histamine injections* The lethal dose of injected histamine is also much decreased after adrenalectomy in other species e g the rat (VOEGTLIN and DYER 1924 CRIVELLARI 1927 SCOTT 1928 MARMORSTON GOTTESMAN and GOTTESMAN 1928 and HOUSSAY 1953) The sensitivity was more pronounced with increasing degrees of adrenal insufficiency (PERLA and MARMORSTON GOTTESMAN 1931) The increased sensitivity after adrenalectomy was not limited to histamine but could be demonstrated for various other substances too

WYMAN (1928) concluded that the increased sensitivity to histamine after adrenalectomy in rats was caused by lack of medullary tissue rather than lack of cortical tissue This was not verified by PERLA and GOTTESMAN (1929 1931) who found that injections of an adrenal cortical extract after adrenalectomy restored the sensitivity to histamine approximately to normal whereas adrenaline had little effect and the sex hormones none at all INGLE (1937) confirmed the importance of the adrenal cortex for maintaining the resistance to histamine injections after adrenalectomy He found however that the medulla also played a role

NOBLE and COLLIP (1941) found that rats kept on 0 9% NaCl as drinking fluid for 6 to 8 days after adrenalectomy had an increased lethality to histamine injections which could be decreased but not restored to normal by giving desoxycorticosterone Furthermore they found an increased lethality to histamine injections 14 to 16 days after hypophysectomy which could be

in the later experiments by isotope dilution. A schematic illustration of the principal chemical reactions involved is shown in Figure 1.

SCHAYER *et al.* (1953) found that mainly two enzymes inactivated histamine *in vivo*. Histaminase was the most important histamine inactivating enzyme in the rat since ^{14}C-imidazoleacetic acid (cf. Figure 1) was the main excretory product. The other main enzyme inactivated histamine by adding a methyl group to a nitrogen atom in the imidazole ring (called ring N methylation) thereby forming methylhistamine (SCHAYER and KARJALA 1956). This enzyme has been called histamine methyl tranferase (LINDAHL 1958) and imidazole N methyl transferase (BROWN *et al.* 1959). A review of the "Catabolism of Physiological Quantities of Histamine in Vivo" has been published by SCHAYER (1959).

In view of the previous findings of changes in histaminase activity *in vitro* after adrenalectomy it is of interest to note that SCHAYER *et al.* (1952) reported that "*no significant difference was found between adrenalectomized or sham operated mice in the ability to destroy minute quantities of injected ^{14}C histamine*". This discrepancy with the findings of ROSE and BROWNE may be due to differences in histamine concentrations administered, species differences or variations in experimental procedure. The finding that adrenalectomy did not change the catabolism of injected ^{14}C-histamine in mice was again reported by SCHAYER *et al.* (1953).

Endogenous formation of histamine from the amino acid L-histidine is catalyzed by the enzyme histidine decarboxylase (e.g. WERLE 1941, SCHAYER 1952, WATON 1956).

The formation of histamine *in vitro* can be studied by adding ^{14}C histidine to a tissue and measuring the amount of ^{14}C histamine formed. With this technique SCHAYER *et al.* (1955) and SCHAYER (1956) found that after adrenalectomy skin and lung tissue formed more ^{14}C histamine *in vitro* whereas glandular stomach tissue formed less. *Thus the formation of histamine in vitro is modified after adrenalectomy.* This factor along with the decrease in histaminase activity *in vitro* affords another explanation for the changes in the histamine content of the tissues in the adrenalectomized animal.

To summarize, four different approaches have hitherto been used to study the effects of adrenalectomy on the metabolism of histamine in the rat:

1. the sensitivity to histamine injections
2. *in vitro* studies on the activity of histaminase
3. the content of histamine in tissues
4. the formation of ^{14}C histamine *in vitro*

restored approximately to normal by giving an ACTH extract but not by giving desoxycorticosterone

Thyroidectomy in rats had no effect on the development of adrenal insufficiency and the increased sensitivity to histamine injections after adrenalectomy (WYMAN 1928)

Histaminase is an enzyme that inactivates histamine by oxidative deamination (e g BEST and MCHENRY 1930) Histaminase has also been called diamine oxidase (ZELLER 1938) since it can oxidize several diamines KARADY *et al* (1940) found *a decreased histaminase activity in vitro* in lung tissue of adrenalectomized rats The decrease after adrenalectomy was more pronounced if water was substituted for sodium chloride as drinking fluid The decreased histaminase activity could be restored approximately to normal by giving an adrenal cortical extract TELFORD and WEST (1961) found a decreased histaminase activity *in vitro* in the ileum of adrenalectomized rats given water

A decreased histaminase activity *in vitro* in various tissues after adrenalectomy was also found in cats (HAEGER and KAHLSON 1952) and guinea pigs (KAHLSON *et al* 1953) The decrease in kidney and intestinal histaminase activity *in vitro* in the cat was apparently caused by the release of the enzyme into the thoracic duct lymph (CARLSTEN 1950)

The decreased histaminase activity was thought partly to explain the findings of ROSE and BROWNE (1938) that rats after adrenalectomy had a *decreased ability to eliminate injected histamine* from the blood the elimination could be restored to normal by giving an adrenal cortical extract (ROSE 1939)

ROSE and BROWNE (1941) reported an *increase in the histamine content* of the stomach and small intestine in adrenalectomized rats The increase was larger if the animals were given water as drinking fluid (which caused adrenal insufficiency) instead of saline MARSHALL (1943) GEIRINGER and HARDWICK (1953) HICKS and WEST (1958) BARTLET and LOCKETT (1959) and TELFORD (1963) have reported findings generally in agreement with those of ROSE and BROWNE (1941)

The introduction by SCHAYER (e g 1952 1955) of methods using ^{14}C histidine and ^{14}C histamine provided entirely new means for studying the catabolism and the formation of histamine in vivo as well as in isolated tissue preparations

The catabolism of histamine *in vivo* was studied by SCHAYER and his co workers (e g 1953) who injected small quantities of ^{14}C-histamine into animals The amount of unchanged ^{14}C histamine and its ^{14}C-labelled deriva

tives excreted in the urine was measured using paper chromatography and

Such experiments have shown that the adrenal glands can influence the metabolism and the effects of histamine The decreased histaminase activity *in vitro* after adrenalectomy might be consistent with an increased content of histamine in the tissues and may also explain the less effective elimination of histamine from the blood However, a decreased histamine inactivation could not be demonstrated using ^{14}C labelled histamine (SCHAYER *et al* 1952 1953)

In view of the reported changes in histamine elimination inactivation and formation it was thought of interest to study the urinary excretion of free histamine in adrenalectomized rats Such experiments were therefore started in this laboratory (BJURO and WESTLING 1959 ANGERVALL *et al* 1961, BJURO and WESTLING 1962 1963)

B The urinary excretion of histamine in rats and the changes caused by adrenalectomy

The excretion of histamine in the urine was demonstrated by e g ANREI *et al* (1944) They showed that there were two forms of urinary histamine free and conjugated Conjugated histamine became biologically active after acid hydrolysis and was later shown to be acetylhistamine (URBACH 1949 TABOR and MOSETTIG 1949) Excretion of histamine has been found in all the species investigated

The method of using the amount of free histamine excreted in the urine as an indicator of the liberation of endogenous histamine was applied to rats by WILSON (1954) However histamine excreted in the urine is both of endogenous and exogenous origin (e g SCHAYER *et al* 1954 GADDUM 1956) The exogenous histamine may be present in the food or be formed by decarboxylation of dietary histidine by certain bacteria in the intestines (e g GALE 1946) In both cases histamine is absorbed and part of it may be excreted in the free form If the contribution of exogenous histamine is large the amount of histamine excreted in the urine would obviously give poor information about the endogenous metabolism of histamine SCHAYER *et al* (1954) succeeded in suppressing the formation of exogenous histamine in the intestines by feeding rats antibacterial agents With such a procedure the urinary histamine is entirely of endogenous origin and therefore a better indicator of the formation and release of histamine in the body GUSTAFSSON *et al* (1957) showed that rats kept on a semisynthetic food without histamine did not excrete more free histamine in the urine than germ free rats This indicated that bacterial formation of histamine in the intestines was unimportant with this diet and antibacterial agents were thus not necessary

Bjurö *et al* (1963 *b*) found in rats kept on this food no difference in the amount of ^{14}C histamine excreted in the urine whether ^{14}C-histidine was injected intravenously or given by mouth This provides a further confirmation that bacterial formation of histamine in the intestines does not occur in rats given this semisynthetic food

The amount of free histamine excreted in the urine can be determined by bio assay using the atropinized guinea pig ileum (Barsoum and Gaddum 1935) An advantage of this method is that histamine can be measured by rather simple means in comparison with those required for determination of ^{14}C-histamine

However when evaluating variations in the amount of histamine excreted in the urine changes in the activity of histaminase and the histamine ring N methylating enzyme must be taken into account This difficulty can be overcome partly by giving aminoguanidine in a dose sufficient to inhibit histaminase as shown *in vitro* by Schuler (1952) and *in vivo* by Schayer *et al* (1953)

As mentioned above injected ^{14}C histamine and presumably also non radioactive (endogenous) histamine is inactivated in part by ring N methylation In female rats the proportion of histamine methylated is less than in male rats (Westling 1958) but nevertheless only about 30% of injected ^{14}C histamine is excreted in unchanged form in the urine in female rats given aminoguanidine The remaining 70% can be accounted for largely by ring N methylation Thus changes in the methylation of histamine can obviously alter the urinary excretion of histamine in rats given aminoguanidine Therefore variations in the amount of histamine excreted in the urine cannot be said to reflect parallel changes in the amount of histamine formed or released unless the enzymatic inactivation of histamine is known in each experimental condition

Rather little is known about the relative importance of histamine from various tissues as sources of the histamine excreted in the urine After an intravenous injection of ^{14}C histidine to intact rats the glandular stomach contained more ^{14}C histamine than other organs examined (Bjurö *et al* 1963 *a*) The ^{14}C histamine formed in the glandular stomach showed a high rate of turnover in contrast with e g the skin Thus it seems probable that histamine from the glandular stomach constitutes a large fraction of the histamine excreted in the urine in rats

As mentioned above the present investigation was initiated by observations on the urinary excretion of free histamine in adrenalectomized rats given aminoguanidine It was found that the amount of sodium chloride intake

aminoguanidine This was done to obtain information about the level of activity of histaminase and the ring N methylating enzyme *in vivo* (Chapter 3)

2 *the formation of ^{14}C histamine in vivo* from injected ^{14}C-histidine The amount of ^{14}C histamine was measured in the urine and in various tissues (Chapter 4)

3 *the formation of ^{14}C histamine in vitro* in various tissues formed from intravenously injected ^{14}C histidine (Chapter 4)

In most experiments the findings with ^{14}C-labelled histamine and histidine were correlated with the amount of non radioactive histamine excreted into the urine or present in the tissues

and with this the degree of adrenal insufficiency were of decisive importance for the changes in urinary histamine In the later experiments (BJURÖ and WESTLING 1962 1963) the intake of sodium chloride was therefore controlled Two main changes in the urinary free histamine were observed after adrenalectomy

1 a *progressive decrease* in the amount of histamine excreted in the urine in rats developing adrenal insufficiency when *kept on a sodium free diet*

2 *a sudden and large increase* in the amount of urinary histamine when sodium chloride was given to rats after a period of adrenal insufficiency In such rats the adrenal insufficiency was corrected and with this the urinary histamine increased It remained at a higher than normal level for several days

In rats kept on large amounts of 0 9% NaCl there were no significant changes in the urinary histamine for approximately the first eight days after adrenalectomy but then a progressive increase was observed

It was difficult to reconcile these findings with the previously mentioned changes in the metabolism of histamine after adrenalectomy The decreased histaminase activity *in vitro* reported by other authors could not play a role since aminoguanidine was given to the rats An increased formation of hist amine could explain the increase in urinary histamine but not the decrease observed in rats with adrenal insufficiency

C The present investigation

The object of the present investigation has been to elucidate the effects of adrenalectomy on the metabolism of histamine *In vivo* experiments have been performed using both a non radioactive method for the determination of the urinary excretion of free histamine and methods using *^{14}C labelled* histidine and histamine These were done both before and after adrenalectomy or sham operation on rats given a semisynthetic food free from histamine The food was either free from sodium or supplied with sodium by giving 0 9% NaCl as drinking fluid With these two diets the sodium chloride con sumption and the degree of adrenal insufficiency could to a large extent be controlled and reproduced As in previous experiments (BJURO and WESTLING 1962 1963) female rats were used

The following main problems were studied

1 *the catabolism of injected ^{14}C histamine* as judged by analysis of the urinary excretion of unchanged *^{14}C histamine and its ^{14}C-labelled derivatives* Such experiments were carried out both in rats given and those not given

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1 *the catabolism of injected* ^{14}C *histamine* as judged by *analysis of the* urinary excretion of unchanged ^{14}C-histamine and its ^{14}C-labelled derivatives Such experiments were carried out both in rats given and those not given

Food

A semisynthetic food (GUSTAFSSON *et al* 1957) was used This food consists of approximately 19 5% (w/w) casein 63% wheat starch 10% arachis oil, 4% of a salt mixture (HUBBEL *et al* 1937) and 1% of a vitamin mixture Its content of histamine was less than 0 05 μg/g of potassium chloride approximately 9 mg/g and of sodium chloride approximately 3 mg/g

By not adding sodium chloride to the food the sodium chloride content could be decreased to approximately 0 2 mg/g At 8 a m every day each rat was given 10 g of this food with a low content of sodium chloride To the food was added 10 ml of drinking fluid Thus two different diets were obtained

1 10 g food mixed with 10 ml distilled water In addition distilled water *ad libitum* as drinking fluid This diet is called sodium free" indicated as H_2O in the Tables and the Figures

2 10 g food mixed with 10 ml of a 0 9% NaCl solution which also was used *ad libitum* as drinking fluid This diet is called 0 9% NaCl

The diet was started 4—6 days before the beginning of the experiment At the end of each day the food not consumed was removed from the cup and weighed The amount of water evaporated from the food during twenty four hours was approximately 1 g The food was mixed with drinking fluid so as to prevent spillage

Collection of urine

The urine was collected every 24 hours at 8 a m In some experiments the urine was collected in periods of eight and sixteen hours respectively (from 8 a m to 4 p m and from 4 p m to 8 a m)

The urine was collected in vessels containing 0 5 ml of 10 N hydrochloric acid which brought the pH of the collected specimen to less than 2 This was done to prevent decomposition of histamine and the growth of bacteria which could form histamine from histidine

The volume of the urine was measured and the urine passed through a filter paper into a test tube The collection vessel was washed with approximately 5 ml of distilled water This solution was filtered added to the urine sample and the total volume was measured As a rule the urinary samples were stored at −20 C

Loss of urine

To estimate the loss of urine in the metabolism cage the following experiment was performed At the end of a twenty four hour collection period after

CHAPTER 2

METHODS

ANIMALS AND THEIR CARE

Animals

White female, non pregnant inbred Sprague Dawley rats were used[1] when they were between 2 and 4 months old and weighed from 120 to 180 g Sham operated intact and adrenalectomized rats were chosen so that the body weight was about the same in the different groups at the beginning of the experiment

The experimental series were indicated by a figure The individual rat in each series was marked at random by a letter plus the figure of the series

Cages

The rats were kept individually in metabolism cages or in groups of three to five in storage cages The metabolism cage had a wire mesh (width 3 mm) in the collecting polyethene funnel (diameter 20 cm) for separating the faeces from the urine Special drinking fountains were used (LAZAROW 1954) These fountains do not leak or spill and therefore accurate measurement of the volume of fluid consumed can be made The food and drinking fluid were *easily accessible even to a rat with adrenal insufficiency*

All rats were kept in the same room at a temperature of from 18 to 22 C The electric light was automatically switched on at 8 a m and off at 5 p m This was done to eliminate variations in the duration of natural light during the year

Drinking fluid

The drinking fluid used was either distilled water or 0 9% NaCl (w/v) which was prepared from sodium chloride (reagent grade) and distilled water

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[1] Obtained from AB Anticimex Stockholm

three subcutaneous injections of ^{14}C histamine into each of four rats (G7 H7 E7 and F7 see Table 1) the cage bottom wire mesh and the funnel were carefully washed with 25 to 30 ml of distilled water The amount of ^{14}C-labelled compounds in this fluid was determined in the same way as the amount of total ^{14}C in the urinary samples

The volume of urine excreted varied from 0 9 to 54 9 ml The amount of ^{14}C in twelve urinary samples from these rats corresponded to an average loss of urine of 0 37±0 0 ml and was largely independent of the total urinary volume (see Figure 2)

Injections

All injections were given at the beginning of the ordinary urine collection period (thus close to 8 a m)

Aminoguanidine sulfate in distilled water (20 mg/ml) was injected sub cutaneously once daily in a dose of about 20 mg/kg body weight (SCHAYER *et al* 1954 WESTLING 1958)

In the catabolism experiments ^{14}C histamine (see p 18) was given in a dose of 25 or 30 μg (base) as a subcutaneous injection with a long needle to avoid leakage

In the *in vivo* experiments ^{14}C histidine (see p 18) was dissolved in a sodium phosphate buffer (see p 20) to a concentration of 88 μg/ml (12 5 μC/ml) or 177 6 μg/ml (25 0 μC/ml) and was injected into a tail vein About 1 5 ml 5% (w/v) glucose was injected through the same needle to flush in all the ^{14}C histidine

OPERATIONS AND ADRENAL INSUFFICIENCY

Bilateral adrenalectomy (in 94 rats) or sham operation (in 73 rats) was performed in the forenoon during 12 to 15 min of ether anaesthesia A 4 to 5 cm skin incision was made in the dorsal midline and after a gentle dis section a muscular incision was made below the lowest rib on each side The adrenal glands were removed with care in order not to injure the adrenal capsule In the sham operation the adrenal region was not touched

Three rats died from an accidental overdosage of ether but otherwise no rats died from the operation On the day of operation the feeding was post poned until immediately after the end of the operation The rats recovered quickly and started to eat and drink within about half an hour The day of operation was not counted when indicating the time in relation to the opera tion

Certain amounts of sodium chloride in adrenalectomized rats can prevent or postpone signs of insufficiency in adrenalectomized rats In the present

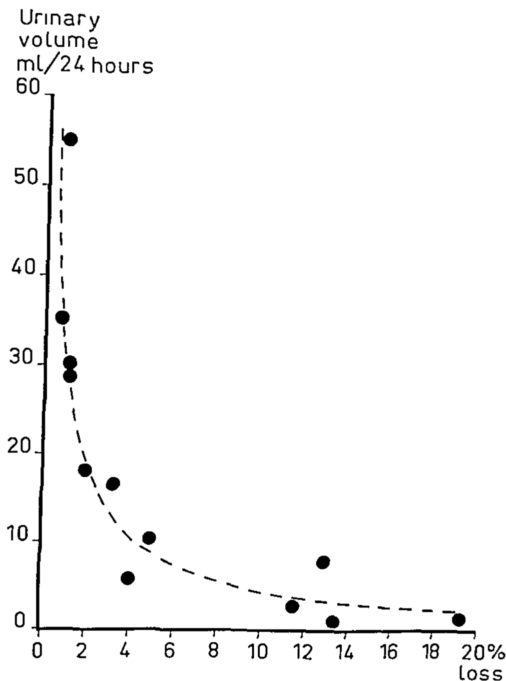


FIG 2 *Loss of urine in the metabolism cage*

The loss in each specimen is expressed as per cent of the volume of urine collected
The dotted line is not mathematically calculated

developed in all rats except those given 0 9% NaCl Apparently accessory adrenocortical tissue is rare in the strain of rats used

As age and initial body weight varied somewhat this might explain the slight variation in the time of onset and the degree of adrenal insufficiency noted

NON RADIOACTIVE HISTAMINE

In the urine

The urinary samples were diluted with Tyrode s solution a dilution of 1 20 was used in rats not given aminoguanidine and 1 40 in rats given amino guanidine Samples of urines containing a higher concentration of histamine were further diluted

With a few drops of bromthymol blue as indicator the samples were adjusted with sodium hydroxide to a pH of approximately 7 4

In the tissues

Extraction of free histamine was performed according to the method described by CODE (1937)

Assay of histamine

Histamine was assayed using the isolated guinea pig ileum as described by BARSOUM and GADDUM (1935) and modified by CODE (1937) Reviews of the determination of non radioactive histamine have been published by e g CODE and MCINTIRE (1956) and CROSSLAND (1961)

The histamine doses were injected into the bath at 1 min intervals which were indicated by an electro mechanical timer This apparatus also automatically washed the two cups with Tyrode s solution by activating solenoid valves (BJURO to be published) The volume of each cup in the organ bath was 4 ml the temperature 34 5 C and the Tyrode s solution was kept aerated by a steady stream of air bubbles

The composition of Tyrode s solution was (g/l) NaCl 8 0 KCl 0 2 $CaCl_2$ 0 $MgCl_2$ 0 1 $NaHCO_3$ 1 0 NaH_2PO_4 0 05 Atropine sulfate 0 1 mg/l and glucose 0 1 g/l were regularly added The glucose increased the sensitivity of the guinea pig ileum

Several consecutive samples of urine from each rat were usually assayed in series This made the determination of small changes in the amount of urinary histamine more reliable As a control samples of special importance were assayed a second time after having been kept frozen for some days or weeks

experiments the amounts of sodium chloride consumed by the adrenalectomized rats given 0 9% NaCl was of the same magnitude as reported by ANDERSSON *et al* (1940 *a*) (from 650 to 940 mg NaCl/day) to be optimal for maintaining life In a companion publication they observed that such a consumption of sodium chloride (by giving 1% NaCl as drinking fluid) also prevents the increase in the amount of radioactive sodium and the decrease in radioactive potassium excreted in the urine by rats with adrenal insufficiency (ANDERSSON *et al* 1940 *b*) Though such rats are still very sensitive to e g cold and starvation it is evident that the effects of adrenalectomy in some respects can be compensated for by the intake of sodium chloride

In the present experiments adrenal insufficiency was said to appear when a rat did not finish its food This occurred from approximately the fourth day after adrenalectomy and on the sixth day they ate an average of 6 3±0 2 g of solid food (cf *Experiment C*)

The degree of adrenal insufficiency was judged from the decrease in body weight and the decrease in the intake of food In addition rats developing adrenal insufficiency showed increasing lassitude muscular weakness coldness and diarrhea Rats with moderate or severe adrenal insufficiency survived if given adequate amounts of sodium chloride This was not the case in rats with extreme adrenal insufficiency which ate and drank very little

The volume of urine excreted by the rats kept on the sodium free diet was 12 5±2 4 ml/24 hours After adrenalectomy the volume of urine was increased for three to four days and then gradually decreased On the sixth day the value was 11 6±1 2 When these rats in severe adrenal insufficiency were given 0 9% NaCl on the seventh day onwards the volume of urine increased to 59 8±6 4 ml but after one day fell to values between 17 6 and 24 6 ml/24 hours (cf BJURO and WESTLING 1963) In rats with extreme adrenal insufficiency the volume of urine was much decreased (see p 31)

Regarding the decrease in body weight in rats with adrenal insufficiency see Figure 7 and *Experiment C*

Where the experimental design permitted all adrenalectomized rats were put on a sodium free diet at the conclusion of the period of observation All 22 rats (see Tables 1—4) treated in this manner died after two to four days from adrenal insufficiency and this proved that no adrenocortical tissue of functional importance remained in these rats In 51 rats used for the purpose of determining the formation of ^{14}C histamine *in vitro* or *in vivo* (Tables 5 and 6) adrenal insufficiency regularly appeared when the rats were kept on the sodium free diet for more than four days after adrenalectomy In 21 rats (Chapter 4) kept on 0 9% NaCl adrenal insufficiency did not develop

Thus approximately four days after adrenalectomy adrenal insufficiency

developed in all rats except those given 0 9% NaCl Apparently accessory adrenocortical tissue is rare in the strain of rats used

As age and initial body weight varied somewhat this might explain the slight variation in the time of onset and the degree of adrenal insufficiency noted

NON RADIOACTIVE HISTAMINE

In the urine

The urinary samples were diluted with Tyrode s solution a dilution of 1 20 was used in rats not given aminoguanidine and 1 40 in rats given amino guanidine Samples of urines containing a higher concentration of histamine were further diluted

With a few drops of bromthymol blue as indicator the samples were adjusted with sodium hydroxide to a pH of approximately 7 4

In the tissues

Extraction of free histamine was performed according to the method described by CODE (1937)

Assay of histamine

Histamine was assayed using the isolated guinea pig ileum as described by BARSOUM and GADDUM (1935) and modified by CODE (1937) Reviews of the determination of non radioactive histamine have been published by e g CODE and MCINTIRE (1956) and CROSSLAND (1961)

The histamine doses were injected into the bath at 1 min intervals which were indicated by an electro mechanical timer This apparatus also automatically washed the two cups with Tyrode s solution by activating solenoid valves (BJURO to be published) The volume of each cup in the organ bath was 4 5 ml the temperature 34 5 C and the Tyrode s solution was kept aerated by a steady stream of air bubbles

The composition of Tyrode s solution was (g/l) NaCl 8 0 KCl 0 2 $CaCl_2$ 0 $MgCl_2$ 0 1 $NaHCO_3$ 1 0 NaH_2PO_4 0 05 Atropine sulfate 0 1 mg/l and glucose 0 15 g/l were regularly added The glucose increased the sensitivity of the guinea pig ileum

Several consecutive samples of urine from each rat were usually assayed in series This made the determination of small changes in the amount of urinary histamine more reliable As a control samples of special importance were assayed a second time after having been kept frozen for some days or weeks

The values for non radioactive histamine are expressed as μg of free histamine base

The identity of the gut contracting substance was checked with mepyramine (an antihistaminic agent) as described by REUSE (1948)

^{14}C LABELLED COMPOUNDS

General

^{14}C histamine (histamine dihydrochloride) and *^{14}C histidine* (L histidine) both labelled in the 2 position of the imidazole ring were obtained from the Radiochemical Centre Amersham England The specific activity of the ^{14}C histamine was 7 25 mC/mM 1 μg base giving 2 500 cpm (counts per minute) under the standardized conditions used (histamine carrier added precipitated as dipicrate and counted at infinite thickness in a gas flow counter)

The ^{14}C histamine used for the subcutaneous injection was dissolved in 0 9% NaCl to a concentration of 100 μg histamine base per ml Glucose was added to a concentration of 0 2% (w/v) (e g SCHAYER and COOPER 1956)

The ^{14}C histidine used had the following specific activities

Experiment A 12 47 mC/mM *experiments B—D* 21 6 mC/mM in the *in vivo experiments E—G* 22 0 mC/mM In the experiment in section I Chapter 4 the specific activity of the ^{14}C histidine was 9 80 mC/mM 1 μg ^{14}C histamine base formed from the ^{14}C histidine should give the following approximate values in our standardized conditions 6 500 11 300 11 500 and 5 100 cpm respectively

To make the values in the *in vitro experiments A—D* directly comparable with each other the values for ^{14}C histamine in *experiment A* were multiplied by 1 73 (21 6 divided by 12 47)

The ^{14}C histidine was dissolved in the sodium phosphate buffer in a concentration of 25 0 or 12 5 μC/ml

The ^{14}C histidine used for the intravenous injections contained a small *amount of ^{14}C histamine as an impurity (cf WHITE 1960) In the experiment* in Chapter 4 section I about 3/4 *of the ^{14}C histamine was removed from the* ^{14}C histidine using butanol and ether extractions The amount of ^{14}C histamine in the injected ^{14}C *histidine corresponded to approximately* 100 cpm Part of this ^{14}C histamine would be excreted in unchanged form and contribute to the figures for ^{14}C histamine in the urine during the first day after the injection *of ^{14}C histidine*

The methods for determining ^{14}C histamine and its ^{14}C labelled derivatives have been described by SCHAYER and others (SCHAYER 1952 SCHAYER and

COOPER 1956 ROTSCHILD and SCHAYER 1958 LINDELL and SCHAYER 1958 KAHLSON et al 1958 WHITE 1959 1960)

To an aliquot (0 5—2 0 ml) of the urine was added non radioactive carrier equivalent to 206 mg of the respective picrate (SCHAYER and KARJALA 1956)

¹⁴C histamine

The sample of urine was made strongly alkaline with NaOH and saturated with Na_2SO_4 Histamine was extracted with *n* butanol and reextracted with HCl evaporated to dryness The histamine dipicrate was prepared and its radioactivity measured By passing the histamine dipicrate through a Dowex 1 (Cl^-) ion exchange column it was converted to histamine dihydrochloride from which the pipsyl derivative (*p* iodobenzene sulfonyl chloride) was prepared

¹⁴C methylhistamine (1 methyl 4 (β aminoethyl) ¹⁴C imidazole)

The urine was made strongly alkaline saturated with Na_2SO_4 and methyl histamine was extracted with chloroform The extract was acidified with ethyl alcohol saturated with dry HCl The methylhistamine picrate was pre pared after evaporation

¹⁴C methylimida oleacetic acid (1 methyl ¹⁴C imidazole 4 acetic acid MeImAA)

The acid urine was passed through a Dowex 50 (H^+) column and the MeImAA was eluted with ammonia The picrate of MeImAA was prepared after evaporation

¹⁴C *imida oleacetic acid* (¹⁴C imidazole 4(5) acetic acid) ImAA)

An aliquot of urine was hydrolyzed in 1 N hydrochloric acid in a sealed glass tube at about 160 C for 5 hours The hydrolysis splits off the ribose conjugated to the ImAA Thus the values given are the sum (total ImAA) of free ImAA and ImAA riboside The ImAA picrate was isolated in the same manner as MeImAA and by passing through a Dowex 1 (Cl^-) column it was converted into ImAA hydrochloride the pipsyl derivative of which was then prepared

Total ¹⁴C in the urine

The total ¹⁴C-activity was measured as described by SCHAYER and COOPER (1956) From the urine suitably diluted with Tyrode s solution two solutions

were prepared 1) 3 0 ml urine+0 6 ml ^{14}C histamine 2) 3 0 ml urine+0 6 ml distilled water From each solution was prepared 5 plates of 0 6 ml each The plates were dried and their radioactivity measured The difference of counts in the plates prepared from the two solutions was due to the added ^{14}C-histamine (internal standard) The amount of total ^{14}C originally present in the sample of urine could then be calculated by proportion

THE FORMATION OF ^{14}C HISTAMINE IN TISSUES

General

The rats were killed by a blow on the head and bleeding through the cut carotid arteries The tissues were removed quickly The stomach was separated from the oesophagus (at the cardia) and the duodenum (at the pyloric sphincter) and opened by cutting along the lesser curvature The glandular stomach (fundus and antrum) was separated from the squamous stomach (forestomach) by cutting slightly oral to the marginal ridge The gut used was the upper part of the jejunum The skin specimen was taken from the abdomen

The tissue specimens were blotted with a filter paper but were not washed since this could influence their electrolyte composition They were then put in beakers diced (about 1 mm cube) and weighed to the nearest 0 01 g The approximate wet weights of the tissues used in the *in vitro* experiments were glandular stomach 0 4—0 6 g jejunum and skin 1 g lung 0 3—0 4 g kidney 0 6 g, liver 0 9—1 g In the *in vivo* experiments the wet weights were glandular stomach 0 7—0 8 g jejunum 3—4 g skin 1 g lungs 1 2—1 3 g kidneys 1 2—1 4 g liver 4—5 g

In vitro

To the tissue specimens was added 1 0 ml (2 0 ml for the skin) of a 0 1 M sodium phosphate buffer with a pH of 7 4 The composition of the buffer was 1 9 ml 0 1 M NaH_2PO_4 8 1 ml 0 1 M Na_2HPO_4 (GOMORI 1955) to which 20 mg glucose was added The sodium concentration of the buffer was 181 mEq/l To the tissue specimens were further added 0 10 ml 10^{-3} M aminoguanidine to inhibit histaminase and 0 10 ml (12 5 μg) ^{14}C histidine In each incubation two samples of liver were used as blanks About 10 to 15 min before adding ^{14}C histidine 0 5 ml 10^{-1} M semicarbazide was added to these samples to inhibit histidine decarboxylase

The beakers with the tissue specimens were put in a shaking incubator (water bath) in a nitrogen atmosphere at 37 C for 3 hours After this incubation 66 4 mg non radioactive histamine dihydrochloride (50 mg base) dissolved in 1 0 ml distilled water was added as carrier 50 mg non radioactive

L-histidine monohydrochloride dissolved in 1.0 ml distilled water was added to dilute the ^{14}C-histidine to a similar specific activity as the ^{14}C-histamine. Perchloric acid was added to a final concentration of 0.4 N to precipitate enzymes and other proteins. The sample was allowed to stand for at least 30 min and then it was ground with sand in a mortar and passed through a filter paper. Perchloric acid 0.2 N was used for washing. ^{14}C-histamine was extracted from the filtrate as described above with the addition of an extraction to reduce the amount of ^{14}C-histidine in the sample. This was made by shaking a butanol fraction with 20 mg non-radioactive L-histidine monohydrochloride.

In vivo

The tissues specimens taken out after the intravenous injection of ^{14}C-histidine were placed in the sodium phosphate buffer. 0.10 ml 10^{-3} M amino guanidine and 1.0 ml of histamine-histidine carrier was added. Extraction and determination of the amount of ^{14}C-histamine was carried out as described above.

MEASUREMENT OF ^{14}C RADIOACTIVITY

The ^{14}C radioactivity in the picrate or pipsyl compounds was measured at infinite thickness under standardized conditions in a gas flow counter without window. The counter was operated in the proportional region, the gas used was methane at atmospheric pressure. The geometrical efficiency of the counter was about 50%. The background activity averaged 19.6±0.1 cpm. Background and a standard were counted after every 10 to 15 samples. At least 1 000 counts were registered from each sample but it was never counted for less than 3 min. The background and standard were counted in the same manner. The average of two counts was used. The background count was subtracted from the observed counting rate. The blank values were subtracted from the figures for formation of ^{14}C-histamine.

The crystalline samples were put on standard planchets with a diameter of 1 inch. Purity was considered achieved when repeated recrystallizations (three to five) with different activated charcoal adsorbents caused no significant change in the level of radioactivity. In some samples the amount of substance after repeated recrystallisations was too small to cover the planchet at infinite thickness. Such samples were diluted with non-radioactive carrier and the results corrected accordingly. The counts obtained from the pipsyl derivatives — ^{14}C-histamine and total ^{14}C-imidazoleacetic acid — were recalculated to the corresponding values for their picrates thus allowing for

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CHAPTER 3

THE CATABOLISM OF HISTAMINE

The present investigation started with a combination of two *in vivo* methods *measurement of the amount non radioactive (endogenous) free histamine excreted in the urine and a simultaneous study of the urinary* ^{14}C *labelled compounds excreted after a subcutaneous injection of* ^{14}C *histamine*

Experiments were first performed on rats given aminoguanidine (cf BJURÖ and WESTLING 1963) Similar experiments were then carried out in rats *not* given aminoguanidine to elucidate whether the earlier reported changes in histaminase activity *in vitro* after adrenalectomy (cf Chapter 1) had any significance *in vivo*

The following information was obtained about the effects of adrenalectomy and adrenal insufficiency on the catabolism *in vivo* (*a*) the pattern of catabolism and the effect of aminoguanidine (*b*) the rate of excretion of the ^{14}C-labelled compounds in the urine (*c*) the relation between the amount of non radioactive and ^{14}C labelled histamine excreted in the urine

EXPERIMENTAL PROCEDURES

All the twenty three rats had three subcutaneous injections of 25 or 30 μg of ^{14}C histamine each on the third (or fifth) day before the operation and on the fourth day and the ninth (or tenth) day after the operation In most cases the urine was collected in twenty four hour periods Non radioactive (endogenous) histamine total ^{14}C ^{14}C-histamine ^{14}C-methylhistamine ^{14}C-methylimidazoleacetic acid and ^{14}C-imidazoleacetic acid were determined in the urinary samples The values for total ^{14}C and ^{14}C-labelled compounds are given in per cent of the ^{14}C (injected as ^{14}C-histamine) In the text variations in the amount of non radioactive histamine excreted in the urine are expressed as changes from the value obtained the day before the operation

From the seventh or eighth day after operation the diet was altered in all rats The rats previously kept on the sodium free diet now had 0 9% NaCl and vice versa Thus regarding the intake of sodium chloride the last injection

the small differences in molecular weight In this way the values for ^{14}C histamine and its ^{14}C labelled derivatives were made directly comparable

STATISTICAL

Mean values are given together with the standard error of the mean (MEAN $\pm$S E M) Comparison of the different groups of rats was made by Student's t test (FISCHER 1954) A P value of 0 05 or less was considered significant in these experiments

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of ^{14}C histamine was given under conditions opposite to those during the two preceding injections

The experimental design is depicted in Figures 3 and 4

RESULTS

Rats given aminoguanidine

The first seven rats were given daily injections of aminoguanidine Aminoguanidine inhibits histaminase and the inactivation of histamine (see Figure 1) will then be due mainly to transformation into ring N methyl derivatives (methylhistamine and methylimidazoleacetic acid) The effectiveness of the aminoguanidine given in the present experiments can be judged from the low values for ^{14}C imidazoleacetic acid excreted in the urine (less than 5 5%)

The results are shown in Tables 1 and 3 and Figure 3

Non radioactive histamine and adrenal insufficiency

The development of adrenal insufficiency and the simultaneous changes in the urinary excretion of histamine in the four adrenalectomized rats were the same in principle as those reported by BJURO and WESTLING (1963)

Catabolism of ^{14}C histamine

BEFORE THE OPERATION — The urinary excretion of ^{14}C histamine in four rats given the sodium free diet did not differ from that in three rats given 0 9% NaCl (26 4$\pm$2 0% and 29 7$\pm$1 4% respectively) There was also no difference in the excretion of ^{14}C labelled histamine derivatives

THE FOURTH DAY AFTER THE OPERATION — Moderate adrenal insufficiency developed in the two adrenalectomized rats kept on the sodium free diet and they excreted somewhat less ^{14}C histamine than before the operation The excretion of ^{14}C-labelled derivatives did not change In two adrenalectomized rats kept on 0 9% NaCl the catabolism of ^{14}C histamine did not change Sham operation caused no significant changes in the catabolism of ^{14}C histamine

From the seventh or eighth day the diet was altered in all rats (see Experimental procedures)

THE NINTH OR TENTH DAY AFTER THE OPERATION — Severe adrenal insufficiency developed in the adrenalectomized rats changed to the sodium free diet and as shown in Figure 3 the urinary excretion of ^{14}C histamine was less than before the operation These rats excreted a correspondingly increased amount of ^{14}C methylhistamine and ^{14}C-methylimidazoleacetic acid in the urine

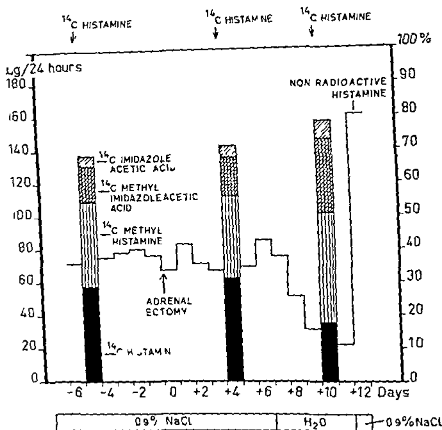


FIG 3 *The catabolism of ¹⁴C histamine in a rat given aminoguanid ne*

Rat C 7 see Table 1 The amount of non radioactive histamine excreted in the urine *is expressed as μg/ 24 hours The amount of ¹⁴C-histamine and its ¹⁴C labelled derivatives* is expressed as percentage of the *injected* ¹⁴C histamine — Note that there is no difference n the catabolism before and after adrenalectomy in a rat not showing adrenal insufficiency (kept on 0 9% NaCl) When the sodium free diet (H_2O) is given, adrenal insufficiency de elops and this is accompanied by a decrease in the amount of both non radioactive and ¹⁴C h stamine excreted in the urine

TABLE 1

Catabolism of ¹⁴C histamine in rats given aminoguanidine

The amounts of ¹⁴C histamine and its ¹⁴C labelled derivatives and total ¹⁴C excreted in the urine within twenty four hours after a subcutaneous injection of ¹⁴C histamine The amounts are expressed in per cent of the *injected* ¹⁴C histamine Non radioactive histamine is given as μg free base/24 hours

H_2O = sodium free diet

0 9% NaCl = 0 9% NaCl as drinking fluid *(and mixed in the food)*

Operation	Day in relation to operation	Drinking fluid	¹⁴C hist amine	¹⁴C methyl hist amine	¹⁴C methyl imidazole acetic acid	¹⁴C imidazole acetic acid	Sum	Total ¹⁴C	μg non radio active histamine
Adrenal ectomy	− 5	NaCl	28 6	25 8	11 0	2 8	68 [illegible]	78	[illegible]0
	+ 4	NaCl	30 9	24 9	11 8	3 4	71 0	98	76
	+10	H_2O	17 3	33 2	24	4 4	[illegible]9 6	78	23
Adrenal ectomy	− 5	NaCl	32 4	24 6	12 5	3 5	73 0	94	84
	+ 4	NaCl	26 6	26 6	11 8	3 1	68 1	94	66
	+10	H_2O	18 2	33 3	24 4	3 6	[illegible]9 5	88	34
Adrenal ectomy	− 5	H_2O	22 0	23 2	14 9	4 4	64 5	91	[illegible]
	+ 4	H_2O	19 4	2[illegible] 5	12 2	2 9	60 0	71	4[illegible]
	+10	NaCl	30	43 4	23 1	5 4	10[illegible] 4	10[illegible]	240
Adrenal ectomy	− 5	H_2O	30 5	20	13 6	3 6	68 4	95	75
	+ 4	H_2O	21 9	26 5	11 1	3 1	6[illegible] 6	93	52
	+10	NaCl	45 5	37 3	19 5	5	107 5	110	336
Sham operation	− 5	NaCl	28 0	23 6	13 4	4 5	69 5	93	63
	+ 4	NaCl	36	22 4	1[illegible] 3	4 3	78	87	71
	+10	H_2O	29 2	[illegible]4 9	18 7	4 6	77 4	84	67
Sham operation	− 5	H_2O	29 1	27 [illegible]	16 8	4 3	77 4	86	9
	+ 4	H_2O	26 7	[illegible]1 5	16 0	3 5	67	75	61
	+10	NaCl	[illegible]9 4	21 6	17 6	4 0	7[illegible] 6	79	6
Sham operation	−	H_2O	24 1	26 6	14 8	4 4	69 [illegible]	80	60
	+ 4	H_2O	25 4	21 0	13 6	3 9	64 8	77	63
	+ 9	NaCl	30 6	2[illegible]	14 5	4 4	71	84	68

Contrary to this the adrenalectomized rats given 0 9% NaCl from the seventh day excreted more ^{14}C-histamine ^{14}C-methylhistamine and ^{14}C methyl imidazoleacetic acid than before the operation The independently determined amount of total urinary ^{14}C was also increased

As can be seen in Tables 1 and 3 the catabolism of ^{14}C histamine did not significantly change in the five sham operated rats This was the case whether they were given the sodium free diet or 0 9% NaCl

Summary

1 Adrenalectomized rats given a sodium free diet and with adrenal insufficiency excreted less unchanged ^{14}C-histamine in the urine than before operation When 0 9% NaCl was given to these rats the adrenal insufficiency was corrected and they then excreted more unchanged ^{14}C histamine in the urine than before operation

2 The amount of ^{14}C histamine excreted in the urine thus changed in the same direction as the urinary non radioactive histamine in adrenalectomized rats in which histaminase was effectively inhibited by aminoguanidine

Rats not given aminoguanidine

In experiments by other authors (cf Introduction) the histaminase activity *in vitro* in various tissues from different species including the rat decreased after adrenalectomy In order to see whether a similar decrease occurred *in vivo* the catabolism of injected ^{14}C-histamine was studied in rats not given aminoguanidine A decreased histaminase activity should cause a decrease in the amount of ^{14}C-imidazoleacetic acid excreted in the urine (see Figure 1)

The results are given in Table 2 Figure 4 shows a typical example The results from the six rats not given aminoguanidine (Table 3) were also used

Non radioactive histamine and adrenal insufficiency

As expected intact rats not given aminoguanidine excreted less non radio active histamine in the urine than intact rats given aminoguanidine (31 8± 1 7 and 67 7±2 9 μg/24 hours respectively) These values are from Tables 1—3 eleven and nine rats respectively

Adrenal insufficiency developed in the five adrenalectomized rats kept on the sodium free diet and the excretion of non radioactive histamine in the urine decreased progressively to an average value of 10 ±2 1 μg on the sixth day after the adrenalectomy These rats were given 0 9% NaCl from the

TABLE 2

Catabolism of ^{14}C histamine in rats not given aminoguanidine

For explanation see Table 1

Operation	Day in relation to operation	Drinking fluid	^{14}C histamine	^{14}C methyl histamine	^{14}C methyl imidazole acetic acid	^{14}C imidazole acetic acid	Sum	Total ^{14}C	µg non radioactive histamine
Adrenalectomy	− 5	NaCl	6 2	5 8	[illegible] 4	60 1	79 [illegible]	79	31
	+ 4	NaCl	8 3	8 5	8 3	[illegible]1 8	76 9	94	34
	+10	H_2O	2 [illegible]	8 0	11 [illegible]	[illegible]4 9	76 3	[illegible]6	11
Adrenalectomy	− 5	NaCl	8 2	7 1	2 9	[illegible]8	76 9	87	40
	+ 4	NaCl	7 6	8 4	4 2	[illegible]7 4	77 6	94	3[illegible]
	+10	H_2O	3 5	7 4	6 9	[illegible]0 1	37 9	38	18
Adrenalectomy	− 5	H_2O	4 8	5 [illegible]	[illegible]	6[illegible] 0	80	83	[illegible]8
	+ 4	H_2O	3 6	4 2	3 [illegible]	64	7[illegible] 6	83	16
	+10	NaCl	1)	—	—	—	—	—	—
Adrenalectomy	− [illegible]	H_2O	[illegible] 6	5 9	[illegible] 8	[illegible]3 1	70 4	80	—
	+ 4	H_2O	[illegible] 9	4 0	3 9	62 4	76	90	24
	+10	NaCl	8 7	10 3	7	63 0	89	94	38
Adrenalectomy	− 3	H_2O	6 6	8 1	6 5	47 1	68 [illegible]	72	[illegible]
	+ 4	H_2O	1 3	7 8	6 0	49 0	6[illegible] 0	68	7
	+ 9	NaCl	9 2	6 4	6 3	[illegible]2 8	74	[illegible]9	40
Sham operation	− [illegible]	NaCl	7 3	9 [illegible]	[illegible]	51	3	8	33
	+ 4	NaCl	10 3	6 8	6 [illegible]	44 4	68 4	81	38
	+10	H_2O	6 8	12 0	10 9	47 6	77 3	90	3
Sham operation	− [illegible]	H_2O	1)	—	—	—	—	—	—
	+ 4	H_2O	12 2	7 1	8 [illegible]	49 4		[illegible]0	[illegible]
	+10	NaCl	12 3	8 2	8 4	[illegible]4 0	82 [illegible]	9	3[illegible]
Sham operation	− 3	H_2O	[illegible] 1	9 5	[illegible]	[illegible]0 [illegible]	74	8	6
	+ 4	H_2O	10 3	[illegible] 6	6 [illegible]	47	7[illegible] 5	81	[illegible]
	+ 9	NaCl	16 1	13	10 8	36 9	[illegible] 0	91	30

nple lost

eighth day Within forty eight hours the adrenal insufficiency was corrected and the excretion of histamine in the urine increased to an average of 91±8 3 µg During the first six days after the adrenalectomy the urinary excretion of histamine in four rats kept on 0 9% NaCl did not change When the sodium free diet was given to these rats adrenal insufficiency developed and the amount of histamine excreted in the urine decreased

Thus the changes in the amount of non radioactive (endogenous) histamine excreted in the urine after adrenalectomy were in principle the same whether or not histaminase was inhibited by giving aminoguanidine

Catabolism of ¹⁴C histamine

BEFORE THE OPERATION — Seven rats kept on the sodium free diet excreted approximately the same amount of unchanged ¹⁴C histamine in the urine as six rats kept on 0 9% NaCl i.e. 6 8±0 ,3% and 8 0±0 65% respectively There was also no difference in the urinary excretion of ¹⁴C labelled histamine derivatives

THE FOURTH DAY AFTER THE OPERATION — Moderate adrenal insufficiency developed in the five adrenalectomized rats given the sodium free diet and the excretion of unchanged ¹⁴C-histamine was slightly less than before the operation but the decrea e was not significant The excretion of ¹⁴C-labelled derivatives did not change significantly In the four adrenalectomized rats kept on 0 9% NaCl the catabolism of ¹⁴C-histamine was unchanged

Four sham operated rats excreted somewhat more ¹⁴C-histamine than before the operation whether given the sodium free diet or 0 9% NaCl The increase was however not significant

The diet was changed in all rats from the sixth or seventh day after the operation

THE NINTH OR TENTH DAY AFTER THE OPERATION — Severe adrenal insufficiency developed in the adrenalectomized rats now given the sodium free diet and the excretion of ¹⁴C-histamine (3 9±0 58%) was significantly less than in unoperated rats on the sodium free diet ($P<0.01$) The adrenalectomized rats now given 0 9% NaCl excreted somewhat more ¹⁴C histamine than before the operation but the increase was not *significant In the* adrenalectomized rats whether given the sodium free diet or 0 9% NaCl the sum of ¹⁴C methyl histamine and ¹⁴C-methylimidazoleacetic acid was somewhat increased but not significantly

The catabolism of ¹⁴C-histamine in five sham operated rats did not significantly change

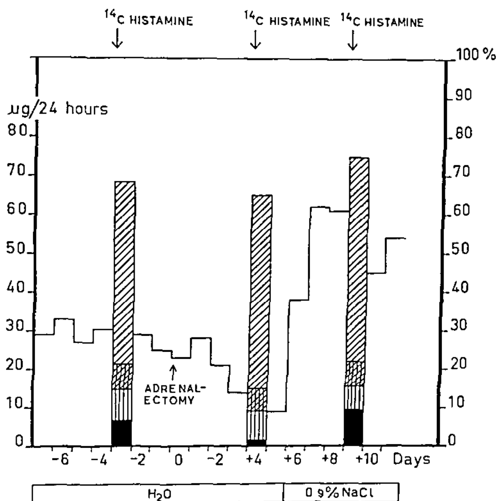


FIG 4 *The catabolism of ^{14}C histamine in a rat not given aminoguanidine*

Rat C 17 see Table 2 For explanation of the symbols see Fig 3 — Adrenal insufficiency develops when the rat is kept on the sodium free diet (H_2O) and there is a decrease in the amount of non radioactive and ^{14}C histamine excreted in the urine

The rate of urinary excretion of total ^{14}C, ^{14}C histamine and non radioactive histamine

As mentioned rats with severe adrenal insufficiency excreted less ^{14}C histamine in the urine than adrenalectomized rats given 0 9% NaCl To see whether this could be caused by a decreased rate of excretion of ^{14}C histamine the daily urine was collected in two fractions during the first eight hours and then for the next sixteen hours after each injection of ^{14}C histamine Due to the

low urinary volumes of rats in severe adrenal insufficiency shorter collection periods were not feasible.

The amounts of total ^{14}C, ^{14}C histamine and non radioactive histamine were determined in the urine samples. Six rats were not given aminoguanidine; of these four were adrenalectomized and two sham operated. Two other sham operated rats were given aminoguanidine. The results are shown in Table 3.

TOTAL ^{14}C. — Before the operation an average of 80% of the injected ^{14}C was excreted during the first eight hours and 17% during the next sixteen hours. Thus most of the injected ^{14}C-histamine could be accounted for by excretion into the urine. This was also the case after adrenalectomy or sham operation in rats given the sodium free diet or 0.9% NaCl with two exceptions. Rat B 12 (see Table 3) with extreme adrenal insufficiency on the tenth day after adrenalectomy excreted only 25% during the first eight hours and an additional 26% during the next sixteen hours. Thus twenty four hours after the injection 49% of the injected ^{14}C histamine was retained in this rat. A similar result was obtained in another rat (B 16, see Table 2) which excreted only 38% of the injected ^{14}C histamine as total ^{14}C in twenty four hours. This rat also showed extreme adrenal insufficiency.

The volume of urine in these two rats was 1.6 and 2.2 ml/24 hours respectively.

^{14}C HISTAMINE. — About 4/5 of the ^{14}C histamine excreted in twenty four hours appeared during the first eight hours after the injection and about 1/5 during the next sixteen hours. A similar distribution of excreted ^{14}C-histamine between the two collection periods was seen also in rats with severe adrenal insufficiency, in which the amount of ^{14}C histamine excreted in the urine in the total twenty four hour period was very small.

In two rats given aminoguanidine the excretion rate of ^{14}C-histamine did not change after sham operation whether they were given the sodium free diet or 0.9% NaCl.

NON RADIOACTIVE HISTAMINE. — The rate of urinary excretion (in $\mu g/h$) of non radioactive (endogenous) histamine was about 50% higher during the first eight hours of the entire twenty four hour collection period. This was the case also after adrenalectomy whether the rats were given the sodium free diet or 0.9% NaCl.

Summary

1. The changes in the urinary excretion of non radioactive histamine in rats not given aminoguanidine were in principle the same as in the rats given aminoguanidine.

TABLE 3

The rate of excretion of subcutaneously injected ¹⁴C histamine

Amounts of ¹⁴C histamine and total ¹⁴C excreted in the urine within the first eight hours and the following sixteen hours after the injection of ¹⁴C histamine For explanation see Table 1 Aminoguanidine was given to rats F12 and G12

Rat	Operation	Day in relation to operation	Drinking fluid	% ¹⁴C histamine			Non radioactive histamine μg			% Total ¹⁴C		
				8h	16h	Sum	8h	16h	Sum	8h	16h	Sum
A12	Adrenal ectomy	− 5	NaCl	[illegible] 1	1 6	6	—	—	—	—	23	—
		+ 4	NaCl	[illegible] 3	1 2	8 5	16	25	41	103	18	1[illegible]1
		+10	H_2O	4 [illegible]	0 5	4 [illegible]	9	14	23	83	29	11[illegible]
B12	Adrenal ectomy	− 5	NaCl	7 [illegible]	2 [illegible]	10 5	14	2[illegible]	39	[illegible]6	14	100
		+ 4	NaCl	[illegible] 0	1 5	9 4	17	22	39	[illegible]	1[illegible]	100
		+10	H_2O	1 [illegible]	0 [illegible]	2 4	6	7	13	2[illegible]	26	51
C12	Adrenal ectomy	− 5	H_2O	6 [illegible]	2 2	8	15	1[illegible]	30	74	17	91
		+ 4	H_2O	5 6	1 4	7 0	8	18	26	88	14	10[illegible]
		+10	NaCl	6 [illegible]	1 1	7 8	1[illegible]	16	31	96	13	109
D12	Adrenal ectomy	− 5	H_2O	4 0	0 9	4 9	10	1[illegible]	2[illegible]	7[illegible]	1[illegible]	9
		+ 4	H_2O	4 4	0 9	5 3	10	18	28	89	14	103
		+10	NaCl	9 9	1	11 1	20	28	48	96	1[illegible]	111
E12	Sham operation	− 5	H_2O	7	2 [illegible]	9 9	17	20	37	[illegible]6	27	103
		+ 4	H_2O	7 3	3 3	10 6	1[illegible]	21	36	76	29	10[illegible]
		+10	NaCl	8	1	10 4	20	21	41	10[illegible]	18	1[illegible]
H12	Sham operation	− [illegible]	NaCl	8 0	1 0	9 0	17	19	36	89	10	99
		+ 4	NaCl	8 9	0 9	9 8	19	19	38	8[illegible]	1	9
		+10	H_2O	6 [illegible]	1 [illegible]	8 1	1[illegible]	16	31	89	11	100
F12	Sham operation	− [illegible]	H_2O	28 6	[illegible] 6	34 2	32	40	[illegible]	82	19	101
		+ 4	H_2O	38 9	8	47 6	30	38	68	8[illegible]	[illegible]6	111
		+10	NaCl	4[illegible] [illegible]	[illegible] 3	[illegible]1	36	39	7	78	18	96
G12	Sham operation	− [illegible]	NaCl	46 [illegible]	4 9	51 4	33	36	6[illegible]	7[illegible]	—	—
		+ 4	NaCl	39 [illegible]	7 1	46 9	34	4[illegible]	76	81	—	—
		+10	H_2O	37 8	4 [illegible]	42 1	31	34	6	71		—

2 Adrenalectomized rats given the sodium free diet and in severe adrenal insufficiency excreted significantly less ^{14}C histamine than before operation This decrease could be prevented by giving 0 9% NaCl The excretion of ^{14}C histamine thus changed in the same direction as the excretion of non radioactive histamine

3 In extreme adrenal insufficiency the excretion of total ^{14}C but not that of ^{14}C-histamine was delayed

4 The rate of urinary excretion of endogenous histamine was higher in day time both before and after adrenalectomy

The relation between the amount of non radioactive (endogenous) histamine, the amount of ^{14}C histamine excreted in the same sample of urine and the degree of adrenal insufficiency

As mentioned rats with adrenal insufficiency excreted less non radioactive histamine and less ^{14}C histamine than before the operation Giving 0 9% NaCl to these rats caused an increase in the amount of non radioactive histamine and ^{14}C histamine excreted in the urine

Both before and after adrenalectomy as can be seen in Figure 5 the changes in the amount of non radioactive histamine were approximately proportional to the changes in the amount of ^{14}C histamine excreted in the urine (nine rats not given aminoguanidine cf Tables 2 and 3)

Figure 6 shows that a similar parallelism occurred in adrenalectomized rats given aminoguanidine (Table 2) except in two rats (E 7 F 7) They excreted a very large amount of non radioactive histamine on the tenth day after adrenalectomy (given 0 9% NaCl) but the amount of ^{14}C-histamine excreted did not increase proportionally

Comparison of Figures 5 and 6 indicates that aminoguanidine increases the amount of unchanged ^{14}C histamine more than it increases the amount of unchanged non radioactive histamine excreted into the urine

Figure 7 shows that there was a relation between the degree of adrenal insufficiency and the decrease in the urinary excretion of unchanged ^{14}C-histamine The rats with extreme adrenal insufficiency as judged from the decrease in body weight excreted the least amount of ^{14}C-histamine

Summary

The present results indicate a close relation between the increase in adrenal insufficiency and the decrease in the urinary excretion of endogenous histamine and unchanged ^{14}C histamine

TABLE 3

The rate of excretion of subcutaneously injected ^{14}C histamine

Amounts of ^{14}C histamine and total ^{14}C excreted in the urine within the first eight hours and the following sixteen hours after the injection of ^{14}C histamine For explanation see Table 1 Aminoguanidine was given to rats F12 and G12

Rat	Operation	Day in relation to operation	Drinking fluid	% ^{14}C histamine			Non radioactive histamine μg			% Total ^{14}C		
				8h	16h	Sum	8h	16h	Sum	8h	16h	Sum
A12	Adrenalectomy	− 5	NaCl	5.1	1.6	6.7	—	—	—	—	[illegible]3	—
		+ 4	NaCl	7.3	1.2	8.5	16	25	41	103	18	1[illegible]1
		+10	H_2O	4.[illegible]	0.[illegible]	4.[illegible]	9	14	23	83	29	11[illegible]
B12	Adrenalectomy	− 5	NaCl	7.[illegible]	2.8	10.[illegible]	14	2[illegible]	39	86	14	100
		+ 4	NaCl	7.9	1.[illegible]	9.4	17	22	39	88	12	100
		+10	H_2O	1.9	0.5	2.4	6	7	13	2[illegible]	[illegible]6	51
C12	Adrenalectomy	− 5	H_2O	6.5	2.2	8	1[illegible]	1[illegible]	30	[illegible]4	17	91
		+ 4	H_2O	[illegible].6	1.4	7.0	8	18	26	88	14	10[illegible]
		+10	NaCl	6.[illegible]	1.1	7.8	15	16	31	96	13	109
D12	Adrenalectomy	− 5	H_2O	4.0	0.9	4.9	10	1	2[illegible]	75	17	9
		+ 4	H_2O	4.4	0.9	5.3	10	18	28	[illegible]9	14	103
		+10	NaCl	9.9	1	11.1	[illegible]0	28	48	96	15	111
E12	Sham operation	− 5	H_2O	7	2.[illegible]	9.9	17	20	37	76	[illegible]	103
		+ 4	H_2O	7.3	3.3	10.6	1[illegible]	21	36	76	29	10[illegible]
		+10	NaCl	8	1	10.4	20	21	41	10[illegible]	18	1[illegible]
H12	Sham operation	− [illegible]	NaCl	8.0	1.0	9.0	17	19	36	89	10	99
		+ 4	NaCl	8.9	0.9	9.8	19	19	38	8[illegible]	12	[illegible]
		+10	H_2O	6	1.0	8.1	1	16	31	89	11	100
F12	Sham operation	—	H_2O	28.6	[illegible].6	34.[illegible]	32	40	7[illegible]	82	19	101
		+ 4	H_2O	38.9	8	47.6	30	38	68	8[illegible]	[illegible]6	111
		+10	NaCl	45.9	5.3	[illegible]1.2	36	39	7[illegible]	78	18	9[illegible]
G12	Sham operation	− [illegible]	NaCl	46.[illegible]	4.[illegible]	[illegible]1.4	33	36	6[illegible]	7[illegible]	—	
		+ 4	NaCl	39.8	[illegible].1	46.9	34	4[illegible]	76	81	—	—
		+10	H_2O	3[illegible].8	4.3	4[illegible].1	31	34	6	[illegible]1		—

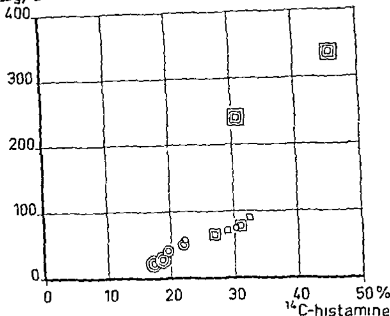


FIG 6 *The rel tion between the amount of non radioactive histamine and ¹ C l istamine excreted in the same sample of urine*

Adrenalectom zed rats given aminoguanidine (see Tal le 1) Symbols as in Figure 5 — Two rats excreted disproport onately large amounts of non radioactive histamine when given 0 9% NaCl at the time of the last injection of ¹⁴C-histamine

DISCUSSION

Confirming previous results in rats with a known intake of sodium chloride (BJURO and WESTLING 1962 1963) these experiments show that after adrenal ectomy two main changes occur in the urinary excretion of non radioactive histamine

1 a progressive *decrease* when adrenal insufficiency develops

2 a large *increase* when sodium chloride is given to rats with adrenal in sufficiency

The present study shows that similar changes in the amount of urinary histamine al o occurred *in adrenalectomi ed rats not given aminoguanidine*

Non-radioactive histamine µg/24 hours

NO AMINOGUANIDINE

50 40 30 20 10 0

0 2 4 6 8 10 12%

^{14}C-histamine

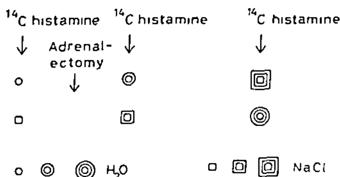


FIG [illegible] *The relation between the amount of non radioactive histamine and ^{14}C histamine excreted in the same sample of urine*

Adrenalectomized rats not given aminoguanidine (see Tables 2 and 3) Below the figure is an explanation of the symbols The circles indicate that the rats were given the sodium free diet (H_2O) and the squares indicate 0 9% NaCl The number of circles and squares refer to the first second and third injection of ^{14}C histamine respectively Observe the change in diet before the last injection of ^{14}C histamine — Note that the two "types of histamine change in the same proportion

The decrease in urinary non radioactive histamine and ^{14}C histamine appeared from about the fourth day after adrenalectomy Thus the absence of the adrenal glands *per se* does not seem to influence the catabolism of injected ^{14}C-histamine This is in accordance with the findings in mice by SCHAYER *et al* (1952)

The *decrease* in the amount of ^{14}C histamine and non radioactive histamine in the urinary samples might be caused by (*a*) loss of urine in the metabolism cage (*b*) retention of histamine in tissues (*c*) a slower absorption or slower excretion of histamine (*d*) a more efficient catabolism of histamine (*e*) a decreased formation of histamine or combinations of these alternatives

(*a*) Loss of urine can at most explain 15–20% of the changes observed (see Methods) For the volumes of urine actually obtained the loss is probably of the order of 2–10%

(*b*) Injected ^{14}C histamine is not bound in the tissues of intact rats (SCHAYER 1952) Whether or not this is the case also in adrenalectomized rats has not been studied specifically in the present work This seems unlikely since the excretion of total ^{14}C in the urine was not significantly decreased except in extreme adrenal insufficiency

(*c*) Rats with adrenal insufficiency have an impaired peripheral circulation In such rats the injected ^{14}C histamine might be more slowly absorbed or excreted It seems reasonable to assume that if a slower absorption or excretion occurred this would also affect the endogenously released histamine

The rate of the urinary excretion of histamine was studied by dividing the daily collection of urine into one period from zero to eight hours and in a second period from eight to twenty four hours after the subcutaneous injection of ^{14}C histamine A slower excretion (or absorption) of the injected ^{14}C histamine might lead to a larger percentage of ^{14}C histamine being *excreted* in the eight to twenty four hour collection period However in rats with adrenal insufficiency the amount of unchanged ^{14}C histamine (as per cent of injected ^{14}C histamine) was decreased in both urinary *portions* by the same proportion This indicates that the decrease in the percentage of ^{14}C histamine cannot be explained *only* by a slower absorption or slower excretion of the injected ^{14}C histamine However it should *be noted that* a slower excretion of ^{14}C labelled histamine derivatives occurred in two rats with extreme adrenal insufficiency A slower absorption or *slower excretion* is likely to give more time for the catabolizing enzymes to inactivate histamine and can thus contribute to the observed decrease in the percentage of ^{14}C histamine excreted in the urine

(*d*) The decrease in the percentage of ^{14}C histamine was not caused by increased histaminase activity since there was no increase in the excretion

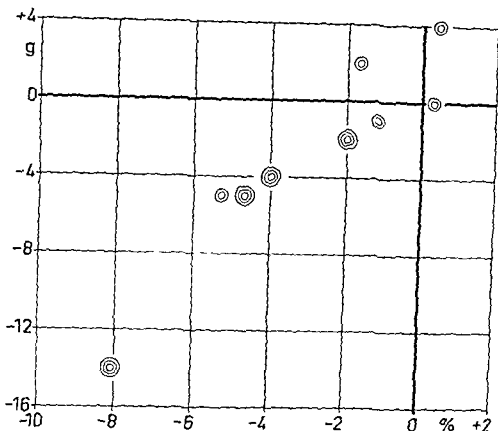


FIG 7 The relation between the changes in body weight (g) and the changes in ^{14}C histamine (%) excreted in the urine

Nine adrenalectomized rats not given aminoguanidine (see Tables 2 and 3) Symbols as in Figure 5 The changes in the percentage of ^{14}C histamine are expressed as the value *before operation minus the value obtained on the fourth or tenth (ninth) day after adrenal*ectomy The change in body weight was calculated similarly — Note that the greater degree of adrenal insufficiency the smaller is the amount of ^{14}C histamine excreted in the urine

TELFORD (1963) claims that *there were no changes in the amount of urinary* histamine in female adrenalectomized rats not given aminoguanidine whether kept on water or 0 9% NaCl as drinking fluid However nothing was reported about the sodium chloride intake or presence of adrenal insufficiency in these rats

Rats given the sodium free diet and with adrenal insufficiency excreted less ^{14}C histamine than before adrenalectomy This decrease appeared to be parallel to the development *of adrenal insufficiency and to the decrease in the urinary excretion of non radioactive* histamine in the same sample of urine

The decrease in urinary non radioactive histamine and ^{14}C-histamine appeared from about the fourth day after adrenalectomy Thus the absence of the adrenal glands *per se* does not seem to influence the catabolism of injected ^{14}C-histamine This is in accordance with the findings in mice by SCHAYER *et al* (1952)

The *decrease* in the amount of ^{14}C-histamine and non radioactive histamine in the urinary samples might be caused by (*a*) loss of urine in the metabolism cage (*b*) retention of histamine in tissues (*c*) a slower absorption or slower excretion of histamine (*d*) a more efficient catabolism of histamine (*e*) a decreased formation of histamine or combinations of these alternatives

(*a*) Loss of urine can at most explain 15—20% of the changes observed (see Methods) For the volumes of urine actually obtained the loss is probably of the order of 2—10%

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(*c*) Rats with adrenal insufficiency have an impaired peripheral circulation In such rats the injected ^{14}C-histamine might be more slowly absorbed or excreted It seems reasonable to assume that if a slower absorption or excre tion occurred this would also affect the endogenously released histamine

The rate of the urinary excretion of histamine was studied by dividing the daily collection of urine into one period from zero to eight hours and in a second period from eight to twenty four hours after the subcutaneous injection of ^{14}C histamine A slower excretion (or absorption) of the injected ^{14}C histamine might lead to a larger percentage of ^{14}C histamine being excreted in the eight to twenty four hour collection period However in rats with adrenal insuffi ciency the amount of unchanged ^{14}C histamine (as per cent of injected ^{14}C-histamine) was decreased in *both* urinary portions by the same proportion This indicates that the decrease in the percentage of ^{14}C histamine cannot be explained *only* by a slower absorption or slower excretion of the injected ^{14}C-histamine However it should be noted that a slower excretion of ^{14}C labelled histamine derivatives occurred in two rats with extreme adrenal insufficiency A slower absorption or slower excretion is likely to give more time for the catabolizing enzymes to inactivate histamine and can thus con tribute to the observed decrease in the percentage of ^{14}C-histamine excreted in the urine

(*d*) The decrease in the percentage of ^{14}C-histamine was not caused by increased histaminase activity since there was no increase in the excretion

of ^{14}C imidazoleacetic acid (and its riboside) — the final products of histaminase action Moreover, a decrease in ^{14}C histamine and non radioactive histamine also occurred in rats given aminoguanidine in a dose that almost completely inhibited histaminase There could also be an increase in the activity of the histamine methylating enzyme since it appears that in some rats the decreased percentage of unchanged ^{14}C histamine in adrenal insufficiency was accompanied by a corresponding increase in ^{14}C methylhistamine and ^{14}C methylimidazoleacetic acid excreted in the urine However, the few animals studied and the variations in the figures make a definite conclusion unjustified

Thus an explanation for the decrease in the urinary excretion of unchanged ^{14}C histamine and non radioactive histamine in adrenal insufficiency might be alternatives *c* and *d* i e a slower absorption or excretion of histamine resulting in a prolonged exposure of histamine to its inactivating enzymes The possibility of a decreased formation of histamine (*e*) is discussed in Chapter 4

Possible explanations for the *increase* in the urinary excretion of non radioactive histamine when 0 9% NaCl was given to rats with adrenal insufficiency are (*f*) a decreased histaminase activity (*g*) a decreased activity of the histamine methylating enzyme, (*h*) release of histamine from the tissues (*i*) an increased formation of histamine

(*f*) A decreased histaminase activity would have caused a decrease in the percentage of ^{14}C imidazoleacetic acid excreted in the urine after an injection of ^{14}C histamine but no such decrease occurred Furthermore the increase *in the excretion of non radioactive histamine* was also seen in rats given aminoguanidine in a dose that almost completely inhibited histaminase

At present it seems difficult to reconcile the findings of a decreased histaminase activity *in vitro* in the lung of the adrenalectomized rat (KARADY *et al* 1940) and in the ileum of the adrenalectomized rat (TELFORD and WEST 1961) with the result of the present study On the basis of observation on the urine *the present study indicates no alteration in histaminase activity after adrenalectomy in the rat*

(*g*) The increase in the urinary excretion of histamine cannot be due to a diminished ring N methylation of histamine since the methylated derivatives were somewhat increased

(*h*) It is possible that the increase in urinary histamine is caused by the release of histamine retained in the tissues during severe adrenal insufficiency The magnitude of the increase and its duration make this explanation unlikely

(*i*) Two rats given aminoguanidine and with adrenal insufficiency were given 0 9% NaCl This caused a disproportionate increase in the amount of non radioactive histamine excreted in the urine as compared with the amount of unchanged ^{14}C-histamine excreted. This suggests that an increased formation of histamine occurred in these rats (see Chapter 4)

The total ^{14}C excreted in the urine could not be fully accounted for by the sum of ^{14}C histamine and its measured derivatives This is in agreement with the findings of several other authors using the same technique The missing radioactivity might be due to ^{14}C acetylhistamine and unknown derivatives of the injected ^{14}C-histamine e g aldehydes which are intermediate steps in the catabolism of histamine (cf SCHAYER 1959)

CHAPTER 4

THE FORMATION OF HISTAMINE

I THE URINARY EXCRETION OF ^{14}C HISTAMINE AND ^{14}C METHYLHISTAMINE IN RATS GIVEN INTRAVENOUS INJECTIONS OF ^{14}C HISTIDINE

^{14}C histamine is excreted in the urine after an injection of ^{14}C L histidine (SCHAYER *et al* 1954) The amount excreted is apparently dependent upon the rate of ^{14}C histamine formation and the rate of its catabolism It is likely that ^{14}C histamine formed endogenously from ^{14}C histidine is inactivated by ring N methylation to a similar extent as ^{14}C histamine given by injection (*BJURO et al* 1963 *b*) *The sum of ^{14}C histamine and ^{14}C methylhistamine* excreted in the urine constitute 50—60% of injected ^{14}C histamine in a female rat given aminoguanidine Determining ^{14}C histamine and ^{14}C methylhistamine excreted in the urine will thus give information about the amount of ^{14}C-histamine formed from the injected ^{14}C histidine ^{14}C methylimidazoleacetic acid would probably be difficult to isolate from other ^{14}C histidine metabolites excreted in the urine of these rats (cf LINDELL and SCHAYER 1958)

EXPERIMENTAL PROCEDURES

Five rats were given *0 9% NaCl* (group *1*) and *four rats the sodium free* diet (group 2) On the fourth day before and on the fourth day after adrenalectomy each rat was given an intravenous injection of 350 *µg* (22 5 *µC*) ^{14}C histidine

RESULTS

In all the rats (cf Figure 8) the first injection of *^{14}C* histidine led to the appearance of *^{14}C* histamine *in the urine The urinary level of ^{14}C histamine* decreased rapidly Rats given the sodium free diet did not differ from those given 0 9% NaCl in the amount of ^{14}C histamine excreted in the urine

On the fourth day after adrenalectomy all rats were given another intravenous injection of ^{14}C histidine As can be seen in Figure 8 the rate of decrease

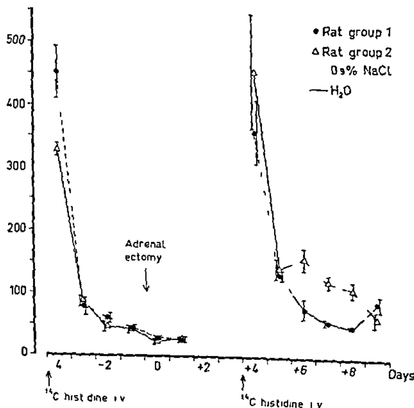


FIG 8 *The a nou t of* ^{14}*C histamine excreted in the urine afte two intravenous injections of* ^{14}*C histidine*

Rats given aminoguanidine The values are expressed as MEAN ± S E M — Note that after adrenalectomy the change fro n the sodium *free diet* (H O) to 0 9 / NaCl caused an increase in the amount of urinary ^{14}C histamine while the change from 0 9 % NaCl to the sod um free d t (H O) caused a decrease in the amount of urinary ^{14}C *histamine*

CHAPTER 4

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In all the rats (cf Figure 8) the first injection of ^{14}C-histidine led to the appearance of ^{14}C-histamine in the urine The urinary level of ^{14}C histamine decreased rapidly Rats given the sodium free diet did not differ from those given 0 9% NaCl in the amount of ^{14}C-histamine excreted in the urine

On the fourth day after adrenalectomy all rats were given another intravenous injection of ^{14}C-histidine As can be seen in Figure 8 the rate of decrease

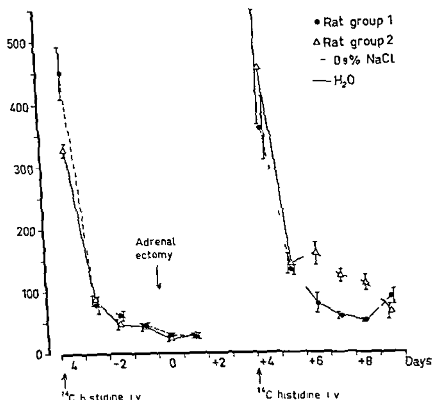


FIG 8 *The amount of ¹⁴C histamine excreted in the u ine after two intravenous injections of ¹⁴C histidine*

Rats g en aminoguanidine The values are expressed as MEAN ± S E M — Note that after adrenalectomy the change from the sodium free d et (H_2O) to 0 9 % NaCl caused an increase in the amount of urinary ¹⁴C-histamine while the change from 0 9 % NaCl to the sodium free di t (H_2O) caused a decrease in the amount of urinary ¹⁴C histamine

CHAPTER 4

THE FORMATION OF HISTAMINE

I THE URINARY EXCRETION OF ^{14}C HISTAMINE AND ^{14}C *METHYLHISTAMINE IN RATS GIVEN INTRAVENOUS* INJECTIONS OF ^{14}C HISTIDINE

^{14}C histamine is excreted in the urine after an injection of ^{14}C-L histidine (SCHAYER *et al* 1954) The amount excreted is apparently dependent upon the rate of ^{14}C histamine formation and the rate of its catabolism It is likely that ^{14}C histamine formed endogenously from ^{14}C histidine is inactivated by ring N methylation to a similar extent as ^{14}C histamine given by injection (BJURO *et al* 1963 *b*) The sum of ^{14}C histamine and ^{14}C methylhistamine excreted in the urine constitute 50–60% of injected ^{14}C histamine in a female rat given aminoguanidine Determining ^{14}C histamine and ^{14}C methylhistamine excreted in the urine will thus give information about the amount of ^{14}C-histamine formed from the injected ^{14}C histidine ^{14}C methylimidazoleacetic acid would probably be difficult to isolate from other ^{14}C histidine metabolites excreted in the urine of these rats (cf LINDELL and SCHAYER 1958)

EXPERIMENTAL PROCEDURES

Five rats were given 0 9% NaCl (group 1) and four rats the sodium free diet (group 2) On the fourth day before and on the fourth day after adrenalectomy each rat was given an intravenous injection of 350 μg (22 5 μC) ^{14}C histidine

RESULTS

In all the rats (cf Figure 8) the first injection of ^{14}C-histidine led to the appearance of ^{14}C histamine in the urine The urinary level of ^{14}C histamine decreased rapidly Rats given the sodium free diet did not differ from those given 0 9% NaCl in the amount of ^{14}C histamine excreted in the urine

On the fourth day after adrenalectomy all rats were given another intravenous injection of ^{14}C-histidine As can be seen in Figure 8 the rate of decrease

groups of rats Changing from 0 9% NaCl to the sodium free diet caused a decrease in the amount of ^{14}C-histamine excreted in the urine whereas the opposite occurred in rats changed from the sodium free diet to 0 9% NaCl (group 1) In the latter group the adrenal insufficiency disappeared

The amount of ^{14}C methylhistamine excreted in the urine on the third day after each injection of ^{14}C histidine was determined in all rats The results together with the amount of ^{14}C-histamine excreted are given in Table 4 On the day before adrenalectomy rats given the sodium free diet (group 2) did not differ from rats given 0 9% NaCl in the amount of ^{14}C methylhistamine excreted in the urine On the seventh day after adrenalectomy however the rats now given 0 9% NaCl (group 2) excreted about three times more ^{14}C-methylhistamine while the rats given the sodium free diet excreted about twice as much ^{14}C-methylhistamine as before operation

It appears that after adrenalectomy the increase in ^{14}C methylhistamine was greater than the increase in ^{14}C histamine

The urinary excretion of non radioactive histamine was greatly increased in the adrenalectomized rats given 0 9% NaCl after a period of adrenal insufficiency This is in agreement with rats E7 and F7 see Table 1

Summary

After an intravenous injection of ^{14}C-histidine the excretion of both ^{14}C histamine and ^{14}C methylhistamine is increased when 0 9% NaCl is given to rats after a period of adrenal insufficiency

II THE FORMATION OF ^{14}C HISTAMINE IN VARIOUS TISSUES *IN VITRO*

The technique developed by SCHAYER (e g 1956) was used in a preliminary attempt to locate the possible increased formation of histamine in rats given 0 9% NaCl after a period of adrenal insufficiency Small amounts of ^{14}C histidine are added to a tissue preparation and the amount of ^{14}C-histamine formed is determined by isotope dilution Since part of the formed ^{14}C histamine may be catabolized to other ^{14}C-labelled compounds the obtained figures are minimum values

EXPERIMENTAL PROCEDURES

In most of the experiments the adrenalectomized rats were kept in metabolism cages Aminoguanidine was given in *experiment A*

TABLE 4

^{14}C histamine and ^{14}C methylhistamine excreted in the urine after two intravenous injections of ^{14}C histidine and the amount of non radioactive histamine excreted in the same samples

The values for ^{14}C histamine and ^{14}C methylhistamine are expressed as *cpm/24 hours*. Non radioactive histamine is given as μg free base/24 hours

Rat	On the third day after							
	the first injection				the second injection			
	Drinking fluid	^{14}C hist amine	^{14}C methyl hist amine	Non radio active hist amine	Drinking fluid	^{14}C hist amine	^{14}C methyl hist amine	Non radio active hist amine
Group 2 A22	H_2O	38	21	60	0.9% NaCl	129	86	184
C22		4[illegible]	39	76		138	121	204
F22		40	29	65		11[illegible]	116	196
G22		—	—	—		89	100	16[illegible]
MEAN ± S.E.M.		41 ±2.0	30 ±[illegible].3	6[illegible] ±4		118 ±11	106 ±8.0	187 ±8.5
B22	0.9% NaCl	—	26	—	H_2O	—	64	—
D22		54	47	78		[illegible]8	44	61
Group 1 E22		39	28	63		69	[illegible]6	[illegible]4
H22		41	22	[illegible]9		44	[illegible]3	42
L22		41	21	6[illegible]		[illegible]6	61	46
MEAN ± S.E.M.		44 ±3.6	[illegible]9 ±4.[illegible]	67 ±4.1		[illegible] ±[illegible].0	[illegible]6 ±3	[illegible]1 ±[illegible]

in the urinary excretion of ^{14}C histamine was similar to that after the first injection of ^{14}C histidine. However the values were greater due possibly to ^{14}C histidine or ^{14}C histamine remaining from the first injection.

The change from the sodium free diet to 0.9% NaCl corrected the adrenal insufficiency and with this there was an increase in the amount of ^{14}C histamine excreted in the urine. The excreted amount remained larger than in the rats given the sodium free diet.

Adrenal insufficiency appeared in rats given the sodium free diet instead of 0.9% NaCl from the sixth day (group 1). They excreted less ^{14}C histamine in the urine than the rats given 0.9% NaCl.

Again on the ninth day after adrenalectomy the diet was changed in both

groups of rats Changing from 0 9% NaCl to the sodium free diet caused a decrease in the amount of ^{14}C-histamine excreted in the urine whereas the opposite occurred in rats changed from the sodium free diet to 0 9% NaCl (group 1) In the latter group the adrenal insufficiency disappeared

The amount of ^{14}C methylhistamine excreted in the urine on the third day after each injection of ^{14}C histidine was determined in all rats The results together with the amount of ^{14}C-histamine excreted are given in Table 4 On the day before adrenalectomy rats given the sodium free diet (group 2) did not differ from rats given 0 9% NaCl in the amount of ^{14}C methylhistamine excreted in the urine On the seventh day after adrenalectomy however the rats now given 0 9% NaCl (group 2) excreted about three times more ^{14}C-methylhistamine while the rats given the sodium free diet excreted about twice as much ^{14}C methylhistamine as before operation

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EXPERIMENTAL PROCEDURES

In most of the experiments the adrenalectomized rats were kept in metabolism cages Aminoguanidine was given in *experiment A*

The rats were fasted the morning they were killed Measurement of ^{14}C-histamine formation *in vitro* from ^{14}C histidine and determination of non radioactive histamine content in the tissues and urine were made as described in Chapter 2

RESULTS

The effect of the sodium free diet and 0 9% NaCl on adrenal insufficiency and the urinary excretion of non radioactive histamine in these rats were in accordance with previous findings in this study and with those of BJURO and WESTLING (1963)

It should be noted that there were multiple punctuate bleedings in the mucosa of the glandular stomach of rats with severe and extreme adrenal insufficiency *The stomachs contained some mucus but as a rule no food*

The results are shown in Table 5 and Figure 9

Experiment A

Twenty five rats were divided into five groups ten rats were adrenalec *tomized, five had the sodium free diet and the other five 0 9% NaCl* Ten rats were sham operated, five of these had the sodium free diet and five had 0 9% NaCl Five intact rats were given the sodium free diet The glandular stomach the skin the lungs the liver and the kidneys were studied

The glandular part of the stomach was used since it has been shown (SCHAYER 1956) *that almost all of the formation of ^{14}C histamine in vitro* in the stomach in normal and adrenalectomized rats is located in this region

As can be seen in Table 5 the glandular stomach of rats in severe or extreme adrenal insufficiency formed a significantly greater amount of ^{14}C histamine *in vitro* (27 900$\pm$6 400 cpm/g) than that of adrenalectomized rats given 0 9% *NaCl (3 400$\pm$1 750* cpm/g) *($P<0$ 01)*

The amounts of ^{14}C histamine formed *in vitro* in the liver and kidney in all rats were not significantly different from the blank activity (10—15 cpm) i e no detectable formation occurred In the skin and lung (average 45 and 400 cpm/g respectively) there were no significant changes in the formation of *^{14}C histamine in vitro after adrenalectomy or sham operation*

The content of non radioactive histamine in the glandular stomach of five rats with severe or extreme adrenal insufficiency was significantly greater (19 3$\pm$2 8 μg/g) than in five adrenalectomized rats given 0 9% NaCl (12 2$\pm$1 1) ($P<0$ 05) In five intact rats kept on the sodium free diet the value was 16 8$\pm$2 0 μg/g *The skin of five rats with adrenal insufficiency* contained more non radioactive histamine (41 4$\pm$2 5 μg/g) than the skin from adrenalectomized rats given 0 9% NaCl (30 4$\pm$3 4) This difference

TABLE 5

The formation of [illegible]C histamine in vitro in the glandular stomach of the rat

The values indicate cpm/g and are expressed as MEAN ± S E M Number within parentheses is the number of rats H_2O – sodium free diet

In eighteen intact rats (given the sodium free diet) used as controls to experiments A–C the value was 3450 ±6[illegible]0 — Note that half of the rats in *experiment A* were kept on H_2O the other half were kept on 0 9% NaCl after the operation. The rats in *experiment D* were kept on H_2O for six days after the operation and on 0 9% NaCl from the seventh day

Experiment	Day after operation	Drinking fluid	Sham operated rats	Adrenalectomized rats
B	[illegible]	H_2O	3 300±7[illegible]0 (5)	2 600±[illegible]0 (8)
	4	H_2O	2 700±3[illegible]0 (5)	4 [illegible]00±610 (8)
	6	H_2O	[illegible] 400±1 0[illegible]0 (5)	9 [illegible]00±670 (8)
C	6	H_2O	[illegible]00±3[illegible]0 (5)	9 100± 300 (6)
A	[illegible]	H_2O	[illegible]000±1 900 (5)	[illegible]7 900±6 400 (5)
		0 9% NaCl	4 [illegible]00±1 650 (5)	3 400±1 [illegible]0 (5)
D	8	0 9% NaCl	4 800±990 (5)	7 800±1 880 (5)
	18	0 9% NaCl	3 300±450 (7)	3 400±830 (6)

was also significant ($P<0.05$) In five intact rats kept on the sodium free diet the value was 32 4±2 5 μg/g The content of non radioactive histamine in the lung did not differ appreciably among the various groups (1 9–2 9 μg/g)

From this experiment it was evident that a large increase occurred in the amount of [14]C-histamine formed *in vitro* in the glandular stomach of rats with severe and extreme adrenal insufficiency This increase could be prevented by giving the rat 0 9% NaCl after adrenalectomy It is possible that minor changes in the amounts of [14]C histamine formed *in vitro* occurred also in other tissues but were masked by the individual variations in the small number of rats in each group

Experiment B

By giving 0 9% NaCl to rats with adrenal insufficiency an increase in the amount of non radioactive histamine excreted in the urine can be elicited from the fourth or fifth day after adrenalectomy (BJURO and WESTLING

1963) Thus it became of interest to see whether the increased formation of ^{14}C histamine *in vitro* in the glandular stomach did appear earlier than on the seventh day after adrenalectomy

Forty seven rats were kept on the sodium free diet They were killed on the second fourth or sixth day after operation together with the intact rats

In the adrenalectomized rats killed on the second day after the operation no significant changes occurred in the formation of ^{14}C histamine *in vitro* in the glandular stomach, whereas on the fourth day a slight and on the sixth day a significant increase occurred ($P < 0.02$)

These results together with the findings in *experiment A* show that in adrenalectomized rats given the sodium free diet (developing adrenal insufficiency) there is a progressive increase in the amount of ^{14}C histamine formed *in vitro* in the glandular stomach This increased formation seems to start from the fourth day after adrenalectomy and seems to be parallel with the increase in adrenal insufficiency

Experiment C

It was found (BJURO and WESTLING 1963) that in sham operated and intact rats smaller amounts of non radioactive histamine were excreted in the urine when the rats were given less food but the decrease was not as pronounced as in the adrenalectomized rats Rats developing adrenal insufficiency spontaneously eat less food which might cause a decrease in the formation of histamine in the glandular stomach (see General discussion) and thus a decrease in the urinary excretion of histamine On the contrary there was an increase in the formation of ^{14}C histamine *in vitro* in the glandular stomach of rats with adrenal insufficiency Therefore it became of interest to see whether the formation of ^{14}C histamine *in vitro* in the glandular stomach was dependent on the amount of food consumed

In a pair feeding experiment sham operated and intact rats were given the same amount of sodium free food as the adrenalectomized rats had eaten the day before Drinking fluid was given *ad libitum* Six adrenalectomized rats were killed on the sixth day after operation and the following day the sham operated and intact rats were killed

The intake of solid food in the adrenalectomized rats decreased from 10 g before operation to an average of 5.8 ± 0.5 g on the sixth day after operation Adrenal insufficiency developed in the adrenalectomized rats with an average decrease in body weight of 9.5 ± 1.5 g on the fifth day after operation as compared to the day before adrenalectomy The restriction of the amount of food to the sham operated and intact rats caused a slowing of their normal weight increase but had no visible influence on their vitality

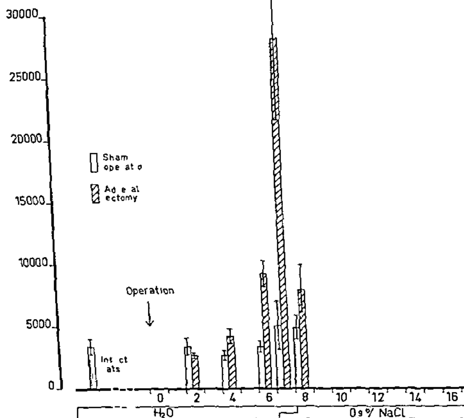


FIG 9 *The formation of C histam ne in vitro n the glandular stomach of the rat*

The line separating H_2O and 0 9 % NaCl is drawn so as to indicate that the rats kill on th seventh day after ope at on were kept on the sodium free diet (H O) and that t rats killed on the e ghth day and eighteenth day were given 0 9 / NaCl from the seven day onwards — Note the progress e increase in the formation of ^{14}C histamine *in vi* n rats developing adrenal insufficiency when kept on the sodium free diet and t decrease wl en 0 9 NaCl is given

The formation of ^{14}C histamine *in vitro* increased in the adrenalectomized rats to approximately the same magnitude as in *experiment B*. In sham operated and intact rats the results were also similar to those in *experiment B*.

Thus it was shown with this experimental procedure that decrease in food intake alone cannot explain the difference in the formation of ^{14}C histamine *in vitro* in the glandular stomach between rats with adrenal insufficiency and sham operated or intact rats.

Experiment D

Severe or extreme adrenal insufficiency develops on the sixth or seventh day after adrenalectomy in rats kept on a sodium free diet. In order to survive the rats then had to have sodium. In rats given 0.9% NaCl from the sixth or seventh day after adrenalectomy adrenal insufficiency can be corrected. This is accompanied by a large increase in the amount of non radioactive histamine excreted in the urine.

Twenty three rats were adrenalectomized or sham operated. The rats were kept on the sodium free diet until the sixth day after operation. From the seventh day after operation 0.9% NaCl was given to all rats. On the eighth and eighteenth day after the operation ten and thirteen rats respectively were killed.

In rats killed on the eighth day after adrenalectomy the amount of ^{14}C histamine formed *in vitro in the glandular stomach was about the same as* that in rats killed on the sixth day after adrenalectomy (cf. *experiment B and C*). However the values were much decreased in comparison with those obtained on the seventh day after adrenalectomy (cf. *experiment A*) which indicates a partial return to normal after the rats were given 0.9% NaCl for one day. Although on the eighth day the values for the adrenalectomized rats were not significantly different from those for the sham operated control rats, *they were still increased in comparison with the values for rats examined* on the second, fourth or eighteenth day after operation.

The adrenalectomized and sham operated rats kept on 0.9% NaCl until the eighteenth day after the operation did not differ in the amount of ^{14}C histamine formed *in vitro* in the glandular stomach. The values were similar to those seen in intact rats.

Summary

1. The amount of ^{14}C histamine formed *in vitro* in the rat glandular stomach progressively increased with increasing degrees of adrenal insufficiency.

2. Giving 0.9% NaCl to a rat with adrenal insufficiency reduced the amount of ^{14}C histamine formed *in vitro* in the glandular stomach.

III THE FORMATION OF ^{14}C HISTAMINE IN VARIOUS TISSUES *IN VIVO*

The observation that the glandular stomach of rats with adrenal insufficiency formed very large amounts of ^{14}C-histamine *in vitro* was not in agreement with the findings of a pronounced decrease in the urinary excretion of non radioactive histamine in these rats To elucidate the formation of histamine in rats with adrenal insufficiency and the effects of sodium chloride in these rats *in vivo* experiments were performed

EXPERIMENTAL PROCEDURES

Rats were killed at various times after they had been given an intravenous injection of ^{14}C histidine and the amount of ^{14}C histamine in the tissues was measured (cf SCHAYER *et al* 1954 SCHAYER and SMILEY 1954)

The adrenalectomized rats were kept in metabolism cages The rats were given no food the day they had an intravenous injection of ^{14}C histidine and they were killed either ten or sixty min after the injection

RESULTS

The effects of the sodium free diet and 0 9% NaCl on adrenal insufficiency and the urinary excretion of histamine were in agreement with previous findings

The results can be seen in Table 6

Experiment E

Of twenty rats kept on the sodium free diet ten were adrenalectomized and ten sham operated On the sixth day after the operation each rat had 88 8 μg (12 μC) ^{14}C histidine injected intravenously Both ten and sixty min after the injection of ^{14}C histidine the glandular stomach of the adrenalectomized rats contained about the same amount of ^{14}C histamine as that of the sham operated rats

Sixty min after the injection of ^{14}C histidine the jejunum of the seven adrenalectomized rats contained more ^{14}C histamine than that of the seven sham operated rats (14 ± 2 0 and 6 ± 0 4 cpm/g respectively) ($P < 0$ 01) The skin of the adrenalectomized rats contained significantly more ^{14}C histamine (1[illegible] ± 2 [illegible] and 6 ± 0 [illegible] cpm/g respectively) ($P < 0$ 01)

The glandular stomach both in the sham operated and the adrenalectomized rats contained more ^{14}C histamine than the other tissues examined This is in agreement with the previous findings of BJURO *et al* (1963 *a*)

TABLE 6

The formation of ^{14}C histamine in vivo in the glandular stomach of the rat

The amount of ^{14}C histamine in the glandular stomach 10 min 60 min and 24 hours after an intravenous injection of 12 5 μC ^{14}C histidine The values are expressed as cpm/g H_2O=sodium free diet 0 9% NaCl=0 9% NaCl as drinking fluid

	Experiment E Six days on H_2O		*Experiment F* Six days on H_2O+one day on 0 9% NaCl		*Experiment G* Six days on H_2O+one day on 0 9% NaCl
Time after injection of ^{14}C histidine	10 min	60 min	10 min	60 min	24 hours
Adrenalectomized rats	329	331	293	361	28
	138	166	662	287	25
	224	155	278	506	29
		287		341	46
		120			
		231			
		244			
MEAN ± S E M	230 ±55	219 ±29	411 ±126	374 ±47	32 ±4
Sham operated rats	105	184	156	166	29
	139	141	128	152	55
	141	671	102	98	28
		128		175	38
		276			
		527			
		129			
MEAN ± S E M	128 ±12	294 ±83	125 ±16	148 ±17	38 ±6 3

Experiment F

Seven rats were adrenalectomized and seven sham operated All rats were given the sodium free diet for six days after the operation and 0 9% NaCl on the seventh day On the eighth day after the operation each rat had an intravenous injection of 12 5 μC ^{14}C histidine

Both ten and sixty min after the injection of ^{14}C histidine the glandular stomach of the adrenalectomized rats contained more ^{14}C histamine than that of the sham operated rats This difference was significant ($P<0.05$) The

jejunum skin lung kidney and liver contained about 5—15 cpm/g not significantly different from the sham operated rats

Experiment G

Four rats were adrenalectomized and four sham operated They were kept on the sodium free diet for six days On the seventh day after the operation the rats were given 0 9% NaCl and 12 5 μC ^{14}C histidine was injected intravenously into each rat Twenty four hours later they were killed

The amount of ^{14}C histamine in the glandular stomach of the adrenalectomized rats did not differ from that in the sham operated rats The values were low in comparison with the values for ^{14}C histamine content ten and sixty min after the injection of ^{14}C histidine (cf *experiment F*) This indicates a high rate of histamine turnover in the glandular stomach in adrenalectomized rats given 0 9% NaCl after six days on a sodium free diet

Summary

1 Rats with adrenal insufficiency and sham operated rats did not differ significantly in the amount of ^{14}C histamine in the glandular stomach ten and sixty min after an intravenous injection of ^{14}C-histidine

2 In adrenalectomized rats given a sodium free diet for six days and 0 9% NaCl for one day the glandular stomach contained significantly more ^{14}C-histamine than that of the sham operated rats at ten and sixty min after an intravenous injection of ^{14}C histidine Twenty four hours after the injection the content of ^{14}C histamine in the glandular stomach was much reduced and there was no difference between the adrenalectomized and sham operated rat This indicates a high rate of histamine turnover

DISCUSSION

Giving 0 9% NaCl to a rat with adrenal insufficiency caused a great increase in the amount of ^{14}C histamine and ^{14}C-methylhistamine excreted in the urine after an intravenous injection of ^{14}C-histidine This was in agreement with the increase in the amount of non radioactive histamine excreted in the urine in these rats Together with the lack of evidence for a decrease in the ring N methylation of histamine (cf Chapter 3) this seems to justify the conclusion that *giving 0 9% NaCl to a rat with adrenal insufficiency caused an increase in the formation of histamine*

To locate the increased histamine formation the *in vitro* method developed by SCHAYER (e g 1956) was used This method is sensitive and the ^{14}C-histamine can be determined independently of

in the tissues This is not the case with the *in vitro* method described by e g WATON (1956) and TELFORD and WEST (1961) in which a large amount of non radioactive histidine is used

Rats with adrenal insufficiency showed progressively decreasing amounts of non radioactive histamine excreted in the urine A paradoxical finding in these rats was the progressive and large increase in the formation of ^{14}C histamine *in vitro* in the glandular stomach Furthermore after an intravenous injection of ^{14}C histidine rats with severe adrenal insufficiency did not differ from the sham operated rats in the content of ^{14}C histamine in the glandular stomach which suggests no change in the formation of histamine

Giving 0 9% NaCl to rats with adrenal insufficiency caused a decrease in the amount of ^{14}C histamine formed *in vitro* in the glandular stomach This was a discrepancy from the significantly increased formation of ^{14}C histamine *in vivo* as judged by the increased amount of ^{14}C histamine in the glandular stomach after an intravenous injection of ^{14}C histidine Furthermore, there was an increase in the amount of endogenous histamine excreted in the urine

Therefore the increased formation of ^{14}C histamine *in vitro* in the glandular stomach of rats with adrenal insufficiency seems not to be representative of conditions *in vivo*

Several factors connected with adrenal insufficiency might be responsible for this discrepancy between the *in vitro* and *in vivo* results Of these the decrease in the amount of sodium (and an increase in potassium) in most tissues of rats with adrenal insufficiency (e g EFRON 1957 PETERS 1959) was of special interest in view of the effects of 0 9% NaCl in rats with adrenal insufficiency

This suggested the possibility that placing the tissue of the glandular stomach (partly depleted of sodium) in a solution with 152 mEq/l of sodium caused an activation of ^{14}C histamine formation Thus an increased formation of ^{14}C histamine would be recorded which is not present *in vivo* A similar mechanism may explain the obtained increased formation of histamine *in vivo* when sodium chloride is given as drinking fluid

Preliminary experiments have shown that smaller amounts of sodium in the buffer (by diluting with isotonic glucose or urea) result in a much decreased formation of ^{14}C histamine *in vitro* in glandular stomach tissue of rats with adrenal insufficiency Decreased amounts of sodium in the buffer also caused a decrease in the formation of ^{14}C histamine *in vitro* in the glandular stomach from the sham operated and intact rats For corresponding values of sodium in the buffer the amounts of ^{14}C histamine formed in the sham operated and intact rats were significantly less than those in the adrenalectomized rats The decreased formation of ^{14}C histamine thus observed might be due to factors

other than the decrease in sodium content For example the solutions used to dilute the buffer might have an inhibitory effect on histidine decarboxylase activity However the similarity in principle between the results obtained with glucose and urea makes this explanation unlikely

SCHAYER *et al* (1955) and SCHAYER (1956) found a decrease in the amount of ^{14}C-histamine formed *in vitro* in the glandular stomach in adrenalectomized rats and an increase in ^{14}C-histamine in the skin and the lung These findings might seem to be different from the observations of the present work However the rats in SCHAYER s experiments were killed on the second day after the adrenalectomy (the day of operation not counted) and probably given a diet containing sodium chloride Thus it is very unlikely that adrenal insufficiency occurred in these rats As can be seen in Table 5 *experiment B* on the second day after the adrenalectomy the glandular stomach formed somewhat less ^{14}C-histamine *in vitro* than that of the intact or sham operated rats This result in rats not showing adrenal insufficiency is thus in accordance with the findings obtained by SCHAYER

TELFORD and WEST (1961) found no change in the formation of non radioactive histamine *in vitro* in the glandular stomach on the fourth day after adrenalectomy in rats given a normal diet and water to drink However methodological differences make a comparison with the present work difficult

As mentioned above the degree of ring N methylation of histamine was somewhat increased after adrenalectomy whether the rats were given the sodium free diet or 0 9% NaCl This is in agreement with the findings in the catabolism experiments (see p 38) Thus the possibility exists that there is an increased ring N methylation after adrenalectomy in the female rat

CHAPTER 5

GENERAL DISCUSSION

(a) *The sensitivity to histamine injections*

Previously several attempts have been made to decide whether the absence of the adrenal medulla or the adrenal cortex causes the observed changes in histamine metabolism after adrenalectomy As mentioned in the Introduction there are different opinions about the protective effect of adrenaline injections upon the increased lethality of histamine given by injection to adrenalectomized rats This might be due to different amount of adrenaline used in rats with varying degrees of adrenal insufficiency However adrenaline seems to offer some protection (e g LECOMTE 1961) The shock that develops when large doses of histamine are injected is probably caused by the profound decrease in arterial blood pressure (e g VOEGTLIN and DYER 1924) due to the vasodilatory action of histamine Thus the protection obtained with adrenaline injections might be due to its vasoconstrictive action

In the present work significant changes in the metabolism of histamine were found to be closely associated with adrenal insufficiency It is well established that after adrenalectomy injections of adrenal cortical hormones can prevent and abolish signs of adrenal insufficiency This is not the case with adrenaline or the adrenal sex hormones

Thus it seems reasonable to assume that the effects of the absence of corticosterone and/or desoxycorticosterone is the cause of the changes in the metabolism of histamine after adrenalectomy Whether it is their mineralocorticoid or glucocorticoid action that is of importance remains to be studied

Previous experiments (BJURO and WESTLING 1960) and unpublished observations suggest that the glucocorticoids but not desoxycorticosterone can prevent the changes in the amount of non radioactive histamine excreted in the urine after adrenalectomy

(b) The catabolism of histamine

As discussed in Chapter 3 the present experiments are not in accordance with the observations of other authors of a decreased histaminase activity *in vitro* in adrenalectomized rats (see Introduction) If such a decrease also occurs *in vivo* it would however seem to be of little importance in the catabolism of histamine as judged from the urinary excretion of unchanged ^{14}C histamine and its ^{14}C labelled derivatives after subcutaneous injections of ^{14}C histamine Moreover the excretion of non radioactive histamine in the urine after adrenalectomy was essentially the same whether or not amino guanidine was given The findings of other authors interpreted by them as indicating a decreased histaminase activity *in vitro* after adrenalectomy might instead be due to a decreased ring N methylation of histamine This explanation however cannot be supported by the *in vivo* catabolism experiments in the present work Thus the biological significance of the decrease in histaminase activity *in vitro* after adrenalectomy in the rat remains unknown

ROSE and BROWNE (1938) found in rats with adrenal insufficiency a decreased rate of disappearance of injected histamine from blood and tissues Since the amounts of histamine used were necessarily large the injections could have caused a further impairment in the circulation in these rats The observed decreased elimination of histamine in these rats might thus have been caused by a circulatory deficiency rather than the postulated decreased histaminase activity Another explanation discussed by SCHAYER *et al* (1953) is that the large amounts of injected histamine "may have been very close to saturation for the histamine metabolizing enzymes in the strain of rats employed

(c) The content of histamine in tissues

A retention of histamine might also explain the repeated observations of an increased content of histamine in most tissues of rats with adrenal insufficiency e g by MARSHALL (1953) HICKS and WEST (1958) The finding of an increased content of histamine in the glandular stomach and skin of rats with severe and extreme insufficiency in the present experiments (cf *experiment* 4 Chapter 4) is thus in accordance with the previous studies of this kind In the present work this increase did not occur in rats with lesser degrees of adrenal insufficiency The increased content of histamine in the tissues was thus probably caused by a nonspecific retention of histamine in the last phase of adrenal insufficiency This explanation is also in agreement with the finding in the present experiments of a retention of ^{14}C-histamine and/or its

^{14}C labelled derivatives in rats with extreme adrenal insufficiency as judged from the decreased urinary excretion of total ^{14}C

Obviously *changes in the formation of histamine (see below)* can contribute to variations in the content of histamine in the tissues

(d) *The urinary excretion of non radioactive histamine*

The amount of non radioactive histamine excreted in *the* urine progressively *decreased* in rats with adrenal insufficiency This decrease seems at least partly to be caused by a more efficient catabolism of histamine which in turn might be due to an impaired peripheral circulation giving more time for contact between histamine and its inactivating enzymes

Adrenal insufficiency disappears in rats when a sufficient amount of sodium chloride is given, and with this there is a large *increase* in the urinary excretion of non radioactive histamine (BJURO and WESTLING 1962 1963) Obviously a contribution to the urinary histamine, during the first one or two days after *giving 0 9% NaCl is likely to come from histamine retained in rats with* severe adrenal insufficiency As discussed previously changes in the level of activity of histaminase are very unlikely Thus neither washing out of retained histamine nor changes in the catabolism of histamine can be the main causes of the increased urinary excretion of non radioactive histamine in these rats It seems likely that changes in histamine formation are of importance for the amount of histamine excreted in the urine

(e) *The formation of histamine*

From the urinary excretion of non radioactive histamine and the catabolism of injected ^{14}C histamine it seems justified to conclude that there is an increased *formation of histamine in rats given sodium chloride after a period* of adrenal insufficiency Such an increase was demonstrated by using two different *in vivo* methods First after an intravenous injection of ^{14}C histidine the simultaneous increase in ^{14}C histamine and ^{14}C methylhistamine excreted in the urine indicated an increase in the formation of histamine Second after an intravenous injection of ^{14}C histidine there was an increase in the amount of ^{14}C histamine in some tissues in rats given sodium chloride after a period of adrenal insufficiency *The increased formation was located mainly* in the glandular stomach This increase in tissue ^{14}C histamine has not been studied beyond the ninth day after adrenalectomy However, it seems unnecessary to suggest an explanation for the prolonged increase in the urinary excretion of histamine other than an increased formation of histamine (cf BJURO and WESTLING 1963)

The findings concerning the formation of ^{14}C-histamine *in vitro* were generally opposite to that which could be expected from the urinary excretion of non radioactive histamine and the formation of ^{14}C histamine *in vivo*. Thus *in vitro* methods used to study the metabolism of histamine in adrenalectomized rats can apparently give misleading results.

An explanation for this discrepancy may be that the glandular stomach of a rat with adrenal insufficiency is partly depleted of sodium. When this tissue is put into a buffer solution (containing sodium) it might take up sodium ions and in doing so increase the formation of histamine. The same mechanism may explain the increased formation of ^{14}C-histamine *in vivo* in the glandular stomach which occurs when a rat with adrenal insufficiency is given a sodium chloride solution as drinking fluid. A support for this suggestion is that the time of the onset of the increase in the formation of ^{14}C histamine *in vitro* in the glandular stomach (see Figure 9) is approximately the same as *in vivo* as the time when 0.9% NaCl can elicit an increase in the urinary histamine in rats kept on a sodium free diet after adrenalectomy (BJURO and WESTLING 1963).

The present experiments do not provide any detailed information about the relation between histamine formation and sodium metabolism. It is of interest to note that histamine has been proposed as a mediator of sodium transport across the cell membrane (EYRING and DOUGHERTY 1955) but experimental evidence for this hypothesis is lacking.

However there may be other causes for the paradoxical increase in the formation of ^{14}C histamine *in vitro* in rats with adrenal insufficiency. It should be emphasized that regardless of the cause the formation of ^{14}C histamine *in vitro* in the glandular stomach of adrenalectomized rats seems to have no counterpart *in vivo*. It should be noted here that an increased formation of ^{14}C histamine measured both *in vitro* and *in vivo* occurred in hyperthyroid rats (BJURO *et al* 1963 a).

(f) *An interpretation of the present work*

In recent years two new hypotheses have been put forward to explain the function of histamine in the body. KAHLSON (for references see KAHLSON 1962) has produced evidence for an intimate association between histamine formation and rapid tissue growth. SCHAYER (for references see SCHAYER 1962) has described experiments in which there was a close connection between histamine formation and certain circulatory reactions e g in response to stressful stimuli.

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It seems premature to try to associate the findings of the present investiga

tion with either of these hypotheses Nevertheless it should be pointed out that an impaired circulation is a cardinal feature of adrenal insufficiency

However, the increased formation of histamine was located mainly in the glandular stomach Therefore the increased formation of histamine might be related to the increased duration and intake of food and drinking fluid

The relation between histamine and the production of gastric secretion has been investigated for a long time CODE (1956) in a review concluded that Stimulation of gastric secretion is a function of histamine No other chemostimulator is interposed between histamine and the parietal cell When starved rats are fed ^{14}C histamine (formed from injected ^{14}C histidine) is released from the glandular stomach and the intestine and ^{14}C histamine is excreted in the urine (SCHAYER and IVY 1957 1958) Another observation that links histamine with gastric activity has recently been made by KAHLSON *et al* (1963) They found that feeding a rat that had been starved for twenty four hours increased the formation of ^{14}C histamine *in vitro* in the glandular stomach This increase reached a peak value 3 to 4 hours after feeding with a return to the fasting value after about 8 hours Moreover from the studies of ^{14}C histidine injections it appears that the greater part of histamine excreted in the urine in intact and hyperthyroid rats comes from the glandular stomach (BJURO *et al* 1963 *a*)

Thus a reason for the changes in the metabolism of histamine after adrenalectomy might be variations in the amount of food and drinking fluid intake with subsequent changes in formation of histamine in the glandular stomach and the gastric secretion rather than the absence of the adrenal glands *per se* The following findings support this suggestion

1 Adrenal insufficiency developing in rats is accompanied by a progressive decrease in the intake of food and in the amount of non radioactive histamine excreted in the urine

2 Intact or sham operated rats given decreased amounts of food also show a progressive decrease in their urinary histamine but this decrease is not as pronounced as in rats with adrenal insufficiency (BJURO and WESTLING 1963) When the ordinary amount of food is given to such intact or sham operated rats there is an increase in the amount of urinary histamine with a return to normal values after about one day

It is well established that changes in gastric secretion occur after adrenalectomy Thus TUERKISCHER and WERTHEIMER (1945) found that adrenalectomized rats had a decreased amount of free and total acid and volume of gastric juice as compared with normal rats These changes could be restored to approximately normal with injections of an adrenal cortical extract but not

with desoxycorticosterone These findings were confirmed by MADDEN and RAMSBURG (1951) and HARTMANN (1956)

Furthermore TUERKISCHER and WERTHEIMER (1945) found a decrease in the amount of pepsin excreted in rats with adrenal insufficiency BAKER and BRIDGMAN (1954) found a decrease in the size of the cells producing pepsin (zymogenic cells) in rats with adrenal insufficiency HIRSCHOWITZ *et al* (1956) found a decrease in the amount of pepsinogen in the stomach of adrenalectomized rats This could be restored to normal by giving hydrocortisone

As mentioned above rats with adrenal insufficiency consume their food and drinking fluid mainly during day time Since the amount of [14]C histamine in the glandular stomach after an intravenous injection of [14]C histidine was determined about twenty four hours after the preceding feeding it is likely that the glandular stomach was in a relatively resting state The formation of [14]C-histamine as judged from the *in vivo* experiments did not differ between the sham operated rats and the rats with adrenal insufficiency However a decrease in the formation of histamine during a period of twenty four hours might have occurred and contributed to the decrease in the urinary histamine in rats with adrenal insufficiency

Adrenalectomized rats have a taste preference for sodium (FREGLY 1958 NACHMAN 1962) Rats given 0 9% NaCl after a period of adrenal insufficiency consume large amounts of this drinking fluid throughout the observation period In analogy this might explain why the formation of [14]C histamine *in vivo* in the glandular stomach was increased although the analyses were made about twenty four hours after the rats were given food

To summarize the present work indicates that the following changes in the metabolism of histamine occur in female rats after adrenalectomy During approximately the first three days after adrenalectomy there are no significant changes in the metabolism of histamine regardless of the intake of sodium chloride After approximately the fourth day progressive adrenal insufficiency develops in rats given the sodium free diet

Parallel with the increase in the degree of adrenal insufficiency there is a decrease in the amount of non radioactive histamine excreted in the urine that might be caused in part by a more efficient catabolism of histamine due to an impaired circulation The progressive decrease in the intake of food in these rats might cause a decrease in the formation of histamine in the glandular stomach contributing to the decrease in urinary histamine

Sodium consumed by these rats can correct the adrenal insufficiency and this is accompanied by a large increase in the urinary excretion of histamine

This increase is caused by an increased formation of histamine mainly in the glandular stomach and might be related to the increased amount of drinking fluid and food consumed by these rats Obviously a contribution to the increased urinary histamine during the first day after the rats had received 0 9% NaCl could be from histamine retained when the rat was in severe or extreme adrenal insufficiency The sustained increase in urinary histamine reported by BJURO and WESTLING (1963) however, cannot have been caused by a "washing out of histamine from the tissues This is so since an increase in urinary histamine also occurred in rats in whom no increase was found in the histamine content of the tissues

CHAPTER 6

GENERAL SUMMARY

The metabolism of histamine has been studied in female rats before and after bilateral adrenalectomy or sham operation Different methods mainly *in vivo* have been used

(a) The urinary excretion of non radioactive histamine

The amount of non radioactive (endogenous) free histamine excreted in the urine decreased progressively when adrenal insufficiency developed in rats kept on a sodium free diet after adrenalectomy In rats given 0 9% NaCl after a period of adrenal insufficiency there was a large and sustained increase in the amount of histamine excreted in the urine

These changes in urinary histamine were in principle the same whether or not histaminase (the main histamine inactivating enzyme in female rats) was inhibited by giving aminoguanidine Thus the changes in the amount of histamine excreted in the urine after adrenalectomy cannot have been caused by variations in the level of activity of histaminase

Sham operation caused no significant changes in the amount of histamine excreted in the urine whether the rats were given the sodium free diet or 0 9% NaCl

(b) The catabolism of small quantities of ^{14}C histamine given by subcutaneous injections

On the fourth day after adrenalectomy there were no significant changes in the catabolism of injected ^{14}C histamine in comparison with the values obtained before operation whether the rats were given the sodium free diet or 0 9% NaCl

Thus the absence of the adrenal glands *per se* seems not to influence the catabolism of injected ^{14}C-histamine

After approximately the fourth day after adrenalectomy progressive adrenal insufficiency developed in rats kept on the sodium free diet Rats with severe adrenal insufficiency showed a significant decrease in the amount

of unchanged ^{14}C histamine excreted in the urine after a subcutaneous injection of ^{14}C histamine This decrease *occurred whether or not histaminase* was inhibited by giving aminoguanidine

Twenty *four hours after the injection of* ^{14}C histamine rats with extreme adrenal insufficiency retained 40–50% of the injected ^{14}C However in rats *with lesser degrees of* adrenal insufficiency there was no decrease in the rate of urinary excretion of unchanged ^{14}C histamine

There was a close correlation between the changes in the amount of non radioactive histamine and the percentage of unchanged ^{14}C histamine excreted in the urine

The decrease in the amount of non radioactive histamine and ^{14}C-histamine excreted in the urine was proportional to the increase in the degree of adrenal insufficiency

The present work indicates that a larger proportion of injected ^{14}C histamine is catabolized in rats with adrenal insufficiency than in intact rats This is due possibly to the impaired circulation in rats with adrenal insufficiency which should give more time for contact between histamine and its inactivating enzymes

The impaired circulation (and decrease in urinary volume) might also explain the retention of histamine that occurs in rats with extreme adrenal insufficiency This is a possible explanation for the increase in the content of tissue histamine found in the present investigation in rats with extreme adrenal insufficiency *but not in rats with* lesser degrees of adrenal insufficiency

There is no evidence in the present work for altered histaminase activity after adrenalectomy in rats

1 *there were no significant changes in the amount of* ^{14}C *imidazoleacetic* acid excreted in the urine after a subcutaneous injection of ^{14}C histamine in adrenalectomized rats *not given aminoguanidine This was the case whether* or not the rats were in adrenal insufficiency

2 the *changes in the amount of non radioactive histamine* and of ^{14}C histamine excreted in the urine after adrenalectomy were essentially the same whether or not histaminase was *inhibited by giving aminoguanidine*

(c) *The formation of histamine in vivo and in vitro*

When rats with adrenal insufficiency are given 0 9% NaCl the amount of non radioactive histamine excreted *in the urine increases* Intravenous injections of ^{14}C histidine to such rats resulted in

1 an increase in the amount of ^{14}C histamine and *^{14}C methylhistamine* excreted into the urine (the rats were given aminoguanidine)

2 an increase in the amount of ^{14}C histamine in the glandular stomach in comparison with sham operated rats or rats with adrenal insufficiency This ^{14}C histamine showed a fast rate of turnover

This indicates *that there is an increase in the formation of histamine in rats given 0 9% NaCl after a period of adrenal insufficiency* This increased formation seems to be located mainly in the glandular part of the stomach

After an intravenous injection of ^{14}C histidine the amount of ^{14}C histamine in the glandular stomach of rats with adrenal insufficiency did not differ from that of sham operated rats This suggests an unchanged formation of histamine in the glandular stomach of rats with adrenal insufficiency but a decrease in the formation of histamine during a period of twenty four hours might have occurred in these rats

In vitro experiments showed that the glandular stomach of rats developing adrenal insufficiency formed increased amounts of ^{14}C histamine and decreased amounts when they had 0 9% NaCl Thus regarding the formation of histamine in the glandular stomach there was a discrepancy between the *in vitro* and the *in vivo* experiments

The formation of ^{14}C-histamine *in vitro* in the glandular stomach after adrenalectomy was generally opposite to that *in vivo* The reason for this discrepancy is not known A possible explanation is that when the glandular stomach of rats with adrenal insufficiency is put into the buffer to incubate it may take up sodium ions and thereby increase the formation of histamine

It should be emphasized that in the present investigation significant changes in the metabolism of histamine were found only in rats with adrenal insufficiency or in rats that had had a period of adrenal insufficiency Changes in the metabolism were not observed during approximately the first three days after adrenalectomy

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THE TRANSPORT OF NONELECTROLYTES IN MUSCLE AS A DIFFUSIONAL PROCESS IN CYTOPLASM

BY

I R FENICHEL AND S B HOROWITZ

UPPSALA 1963

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FROM THE EASTERN PENNSYLVANIA PSYCHIATRIC INSTITUTE PHILADELPHIA U.S.A
AND THE INSTITUTE OF PHYSIOLOGY UNIVERSITY OF UPPSALA SWEDEN

THE TRANSPORT OF NONELECTROLYTES IN MUSCLE AS A DIFFUSIONAL PROCESS IN CYTOPLASM

BY

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UPPSALA 1963

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Introduction

The transport of simple organic nonelectrolytes in cells is a passive process often showing marked chemical specificity. The pattern of this specificity has been elucidated, leading to the widely accepted view that the transport is primarily determined by the permeability of a thin lipid membrane at the cell surface (Collander and Bärlund 1933; Höber 1950, Ch 10). In the simplest formulation of this model chemical specificity arises from the distribution coefficients of nonelectrolytes between lipid and aqueous phases. Such a model roughly accounts for the observed transport but disconcerting features exist:

1. Calculated membrane diffusion coefficients are unusually small (Zwolinski, Eyring and Reese 1949). For example, using data from the present work for the efflux of n-butanol from frog sartorius muscle, a diffusion coefficient on the order of 10 ¹¹ cm /sec can be calculated for a 100 Å membrane. No adequate explanation has been offered for the magnitude of these coefficients which are extraordinarily small for a material that by hypothesis acts as a nonpolar solvent and thus is capable of only weak interactions.

2. As a corollary of membrane permeation models, cellular transport should show a simple exponential time course. However, with the advent of radioisotopic techniques the more complex bulk diffusion kinetics have been demonstrated in both nonelectrolyte (Horowitz and Fenichel 1964) and ion transport (Harris and Prankerd 1957; Ling 1962, Ch 11). Also autoradiographic evidence for bulk diffusion has been provided for sodium (Abelson and Duryee 1949).

3. The pattern of chemical specificity observed in cellular transport can be shown to occur commonly in the diffusion of nonelectrolytes in hydrogen bonding solvents (see Ch 4). Thus the need to invoke a lipid-water distribution process to account for the presence of this pattern in cellular transport is open to question.

CHAPTER 1

Experimental Procedure and General Results

Frog skeletal muscle recommends itself to transport studies for a variety of reasons

1 As observed by Overton (1902) and confirmed in the present study transport in this tissue shows the lipid solubility pattern of chemical specificity

2 The large size of the individual muscle fibers results in a slow flux of materials permitting detailed resolution of the time course of flux

3 The muscle bundle is homogeneous in the dimensions of the individual fibers composing the tissue

4 Muscle fibers are regular in shape and show a degree of micro scopic long range homogeneity and order that is unsurpassed among commonly available cells

5 By virtue of adherent connective tissue muscle bundles may be manipulated with speed and precision without being damaged

6 Frog muscle is durable *in vitro* in a proper Ringer solution it may be kept alive for days

The commonly used sartorius possesses all these virtues but has the disadvantage of being multicellular and containing extensive channels of extracellular space As a result the experimental kinetics represent in a complex manner diffusion in the extracellular space in addition to the desired single fiber flux This complication can be mitigated by using a single fiber or a small bundle of fibers which permits more direct exposure of individual fibers to the bathing solution But small bundle kinetics are themselves complicated by uncertainties such as adherent fluid and extraneous connective tissue—much less control lable than in large tissues—which make the early kinetics difficult to interpret The latter are critical since in all models the flux is ulti mately exponential

There is consequently advantage in using both large and small preparations We have studied both the sartorius and the very small

Each of these points suggests an alternative to lipid membrane permeation models that the properties of nonelectrolyte transport in cells may arise from the nature of the diffusional process in cytoplasm The intent of the present study is to examine the feasibility of this explanation The hypothesis of cytoplasmic diffusion as the rate limiting step will be considered in the light of the major features of the transport the time course the magnitude of the flux coefficients and the specificity

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temperature of the experiment for about two hours Twenty five milliliter portions of washout fluid were placed in small beakers (usually 10 to 12) held in the constant temperature water bath by a plastic frame these came to temperature equilibrium with the bath The muscle was drawn up out of its incubation fluid and grasped at its tendinous insertion with a fresh forceps which was then clamped with a hemostat The muscle was carefully wiped against the inside surface of the incubation flask to drain off excess fluid and then suspended successively in the 25 ml[1] washout solutions for appropriate periods of time Transfer of the muscle between baths took 1 to 1½ seconds The muscle was agitated manually in each bath to assure good mixing of the washout fluid At the conclusion of these wash periods the muscle was placed in a 25 ml portion of washout fluid at room temperature for several hours to recover the remainder of the radioactive material Finally the muscle was blotted briefly on filter paper weighed and tested for exciteability by being placed across the terminals of a 1½ volt dry cell No experiment was considered acceptable in which a muscle underwent change in appearance during the course of the experiment or failed to twitch when stimulated

Extensor longus digiti IV

The procedure with the extensor was similar to that with the sartorius However the fragility of the muscle dictated that manipulation be minimized The following modifications of the above procedure were used

(1) Equilibration times were reduced on the basis of previously determined flux rates in the sartorius

(2) The muscle was held at both ends by fine pointed forceps (Keynes 1954)

(3) The volume of each portion of washout fluid was 7 ml corresponding to the smaller size of the muscle

Sample handling and analysis

A one ml aliquot of each portion of washout fluid and of diluted incubation fluid was pipetted into a 22 ml counting vial with 20 ml of

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[1] This volume dilutes the muscle about 300 fold effectively the concentration of tracer in the washout fluid is always zero

extensor longus digiti IV of *R pipiens* The detailed kinetics in the sartorius are compared with theoretical kinetics for both bulk and membrane models the extensor on the other hand provides direct evaluation of the cellular flux rate constants essentially unmodified by slow extracellular diffusion and permits confirmation of the theoretical treatment of the sartorius

Materials

Muscles from recently captured healthy (green flanked) *Rana pipiens* were used Radioactive material was added to the stock Ringer s solution (Ling 1962) to serve as the incubation fluid washout fluid contained an equal concentration of nonradioactive material Osmotic and concentration dependent effects are thus avoided

Efflux kinetics were obtained for ten compounds members of three homologous series at various temperatures between 0 C and 30 C and in some cases at several concentrations

The labeled compounds with specific activities sources and lot numbers were methanol C^{14} (1 0 mC/mM New England Nuclear Corp NENC 45 290) ethanol C^{14} (2 0 mC/mM NENC 65 64) n propanol C^{14} (0 80 mC/mM NENC 65 13) n butanol C^{14} (2 74 mC/mM NENC 45 163) formamide C^{14} (0 509 mC/mM NENC 65 208) acetamide C^{14} (1 0 mC/mM NENC 42 260) propionamide C^{14} (1 93 mC/mM NENC 71 73) thiourea C^{14} (2 0 mC/mM NENC 65 31) thiourea S^{35} (20 5 mC/mM when received NENC 50 236) thiourea C^{14} (1 77 mC/mM the Radiochemical Center Amersham) methyl thiourea S^{35} (4 15 mC/mM when received Nuclear Chicago Radiochemical Center Amersham NC RC 18168) ethyl thiourea S^{35} (5 04 mC/mM when received NC RC 18169) Non radioactive compounds were all reagent grade or better Merck methanol and *n* butanol Eastman *n* propanol formamide acetamide and propionamide Mallinckrodt thiourea The substituted thioureas were synthesized from Eastman isothiocyanates by the method of Moore and Crossley (1955)

Methods

Sartorius

An isolated sartorius was rinsed in Ringer s solution and placed for a short time in a volume of Ringer s containing nonradioactive compound It was then placed into 3–5 ml of radioactive Ringer s for several hours—never less than three and usually overnight—at 4°C Then the muscle in its equilibration fluid was brought to the

Experimental Results

Three typical efflux curves for the sartorius appear in Fig 1 All of the more than sixty curves obtained in this study are of similar appearance The gross characteristics of these curves are a long pre exponential curved phase followed by an exponential phase which extrapolated to zero time intercepts the ordinate axis generally between 0 45 and 0 60

The kinetic parameters derived from the experimental efflux curves for the sartorius—S the negative slope of the exponential phase of

TABLE 1 *The efflux parameters of 10 nonelectrolytes from R pipiens sartorius*

Compound	Temperature (C)	Muscle weight (mg)	$S \times 10^4$ (sec $^{-1}$)	I
thiourea	1 6	9 8	1 59	64
	1 [illegible]	[illegible]9 3	2 09	5[illegible]
	6 7	71 6	[illegible] 60	[illegible]3
	10 6	75 0	4 17	64
	[illegible]0 5	103 5	5 [illegible]4	63
	[illegible] 3	87 7	7 09	5[illegible]
	9 8	78 4	11 5	60
methyl thiourea	0 8	83 1	4 01	5
	0 8	101 4	2 81	0
	10 6	101 [illegible]	4 83	66
	0 1	80 2	9 56	5[illegible]
	[illegible]0 8	100 [illegible]	7 1[illegible]	63
	9 3	101 3	9 6[illegible]	5
ethyl thiourea	0 7	[illegible]8 5	5 44	59
	0 8	90 3	4 01	5[illegible]
	5	8[illegible] 3	3 98	55
	10 [illegible]	8[illegible] 5	4 6[illegible]	35
	0 [illegible]	88 7	9 56	5[illegible]
	0 [illegible]	71 0	10 [illegible]	48
	21 9	80 9	59	58
	30 0	9 0	1[illegible] 5	48
formamide	10.3	8[illegible]	1 1	38
	10 3	83 [illegible]	10 5	4[illegible]
	9 [illegible]	87 5	34 6	4[illegible]
	9 6	[illegible]8 6	7 4	59

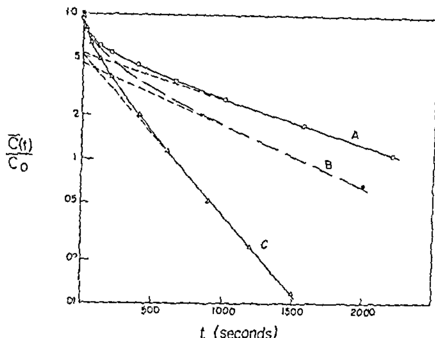


Fig 1 *Efflux of three nonelectrolytes from the sartorius of R pipiens* Ordinate mean fractional tissue concentration Points are experimental curves are fitted Curve A thiourea at 22 3 C Curve B formamide at 10 3 C Curve C ethanol at 7 3 C

scintillation fluid[1] and counted in a 314 EX Packard Liquid Scintillation Spectrometer at 2°C The standard error of the count was generally below 1½ per cent

Data was analyzed as follows Let n_i represent the number of counts per minute less background in a one ml aliquot from the ith washout bath V the volume of a washout bath and N the total number of washout baths Then $V\sum_{i=1}^{N} n_i$ represents the total counts in the muscle at the start of the experiment and $V\sum_{i=1}^{N} n_i/wt$ of muscle represents the *initial total tissue concentration in counts per minute* per gram At the end of the first washout period $V n_1$ counts have left the muscle and so the *mean tissue concentration* at this time is $\bar{C}(t_1) = V\sum_{i=2}^{N} n_i/wt$ Similarly at the end of the jth washout period the mean concentration is $V\sum_{i=j+1}^{N} n_i/wt$ At any time the relative mean concentration $\bar{C}(t_j)/C_0$ is given by

$$\bar{C}(t_j)/C_0 = \sum_{i=j+1}^{N} n_i \Big/ \sum_{i=1}^{N} n_i$$

This is plotted semilogarithmically against time

[1] 4.0 g of PPO (2,5-diphenyloxazole), 50 mg of POPOP (1,4-bis-2-(5-phenyloxazolyl)) benzene) and 150 g of naphthalene in 1 liter of p-dioxane

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thiourea	1 6	97 8	1 [illegible]9	64
	1 [illegible]	59 3	[illegible] 09	5[illegible]
	6 [illegible]	1 6	2 60	[illegible]3
	10 6	7[illegible] 0	4 17	64
	[illegible]0 [illegible]	103 5	5 [illegible]4	63
	[illegible] 3	87 7	7 09	5[illegible]
	[illegible]9 8	[illegible]8 4	11 5	60
methyl thiourea	0 8	83 1	4 01	[illegible]
	0 8	101 4	[illegible] 81	[illegible]0
	10 6	101 [illegible]	4 83	66
	20 1	80 [illegible]	9 56	5[illegible]
	0 8	100 2	7 1[illegible]	63
	29 3	101 3	9 6[illegible]	57
ethyl thiourea	0 7	[illegible]8 5	5 44	[illegible]9
	0 8	90 3	4 01	[illegible]
	7 5	8 3	3 98	55
	10 5	8 5	4 6[illegible]	[illegible]
	[illegible]0 [illegible]	88 7	9 56	[illegible]5
	0 [illegible]	71 0	10 2	46
	[illegible]1 9	80 9	[illegible]8	[illegible]8
	30 0	9 0	1 5	48
formamide	10 3	85	1[illegible] 1	38
	10 3	83 2	10 5	4[illegible]
	9 5	87 5	34 6	4[illegible]
	9 6	[illegible]8 6	27 4	59

Table 1 (*continued*)

Compound	Temperature (°C)	Muscle weight (mg)	$S_e \times 10^4$ (sec^{-1})	I
acetamide	0 8	100 2	8 87	53
	1 1	66 3	7 74	57
	1 1	64 5	11 4	62
	1 3	109 0	9 27	4[illegible]
	19 8	70 5	26 8	40
	19 8	77 3	18 2	52
	22 0	102 1	23 4	58
	22 0	111 0	18 1	55
propionamide	0 6	82 6	11 5	61
	1 0	87 3	10 3	57
	1 0	107 5	10 9	53
	19 8	82 0	20 5	55
	19 8	87 4	21 2	52
	21 7	102 2	19 9	63
	21 8	77 0	20 4	55
methanol	1 5	82 6	23 4	51
	1 5	115 7	21 8	58
	7 4	91 4	31 6	5[illegible]
	20 4	121 8	50 1	6[illegible]
	20 4	95 6	54 8	46
ethanol	1 1	85 1	25 7	56
	1 1	10[illegible] 3	25 9	58
	7 3	88 7	27 2	57
	20 4	85 3	44 3	46
	20 6	80 7	51 1	47
	20 6	97 0	56 5	59
n propanol	0 8	60 8	[illegible]5 3	55
	0 9	95 2	20 4	58
	7 6	86 1	[illegible]6 [illegible]	54
	20 0	99 5	44 3	60
	20 0	67 2	52 9	48
	20 6	88 5	39 7	54
n butanol	0 4	105 4	24 1	55
	0 5	79 1	[illegible] 6	47
	7 4	9[illegible] 6	18 6	47
	7 6	83 5	1[illegible]	48
	[illegible]0 1	71 0	45 [illegible]	55
	[illegible]0 1	85 7	46 2	60
	20 4	96 3	[illegible]5 7	41

S_e is the negative slope of the exponential phase of efflux I is the ordinate intercept

TABLE 2 *The efflux of three nonelectrolytes of similar molecular weight from R pipiens M extensor longus digiti II*

Compound	Temperature ([illegible] C)	[illegible] × 10[illegible] (sec [illegible])
thiourea	1 [illegible]	0 315
	10 4	0 [illegible]45
	19 8	1 94
propionamide	1 2	5 38
	10 5	9 03
	[illegible]0 [illegible]	14 8
n butanol	1 [illegible]	14 5
	1 3	13 1
	1 8	1 [illegible] 5
	10 9	18 4
	11 1	18 9
	20 2	[illegible]8 1

S is the negat[illegible]ve slope of the exponential phase of efflux

efflux and *I* the ordinate intercept—are given in Table 1 The temperature of each experiment and the muscle weight are also provided

Some efflux curves for the extensor are given in Chapter 2 (Figs 5 and 6) The values of *S* obtained in the extensor appear in Table 2

The analysis of these curves and data will be pursued in Chapters 2 and 3

CHAPTER 2

Analysis of the Form of the Efflux Curves

The purpose of this chapter is to attempt to establish the identity of the rate limiting process in the cellular transport of nonelectrolytes In principle this is easily accomplished for fluxes in the individual cell since the kinetics for membrane permeation and bulk diffusion are distinctive In radial bulk diffusion in a long cylinder such as the muscle cell the efflux kinetics are given by (Crank 1956)

$$\frac{\bar{C}(t)}{C_0} = \sum_{j=1}^{\infty} \frac{4}{\gamma_j} \exp\left(-D_c \gamma_j t/r_0\right) \tag{1}$$

when the bathing solution is free of solute $\bar{C}(t)$ is the mean concentration at time t C_0 the uniform initial concentration γ_j the jth root of the Bessel function $J_0(\gamma)=0$ and r_0 the fiber radius The parallel equation for surface permeation is

$$\frac{\bar{C}(t)}{C_0} = e^{-\sigma t} \tag{2}$$

where σ is a surface rate constant for thin membrane of permeability P $\sigma = 2P/r_0$

In a multicellular tissue containing extracellular space however the cellular fluxes are in part regulated by concentration gradients in the extracellular space so that the simple expressions of Eqs 1 and 2 are no longer strictly applicable The more important the contribution of diffusion in the extracellular space the less discernible become the contributions of the cellular processes Nevertheless it will be shown that for nonelectrolyte transport in the sartorius a distinction persists in the overall tissue kinetics which allows the resolution of the rate limiting process Another potential complication of multicellularity heterogeneity of fiber size is discountable so that in the theoretical treatments of the kinetics the fibers in a muscle will be taken to be all of the same diameter This course is justified by several

lines of evidence that the small degree of heterogeneity does not influence the principal features of transport (Creese Neil and Stephenson 1956 Pitts 1954) Direct confirmation of this in both the sartorius (Ling 1962) and the extensor (Keynes 1954) is provided by the strictly exponential kinetics observed in the efflux of potassium

Theoretical Kinetics in the Sartorius

The measured concentration in the sartorius contains three components cellular solute extracellular solute (within the muscle) and carryover solute The latter is extra solute brought from the equilibration fluid to the first washout bath in the fluid adhering to the muscle and in the pieces of connective tissue attached to the ends of the muscle This extra solute is lost from the muscle almost instantaneously once the washout has begun but its presence will affect the mean total tissue concentration as follows Let n_0 represent the total tracer solute molecules in the muscle at the beginning of the washout V V and V the volumes of cellular space extracellular space and carryover space resp and C^0 and C^0 the cellular and extracellular tracer solute concentrations prior to washout Then

$$n_0 = V \; C_c^0 + V \; C^0 + V \; C^0$$

since the concentration of material in V is the same as that in the extracellular space Introducing the distribution coefficient $K = C^0/C^0$ we obtain

$$n_0 = C^0\left(V \; + \frac{V \; + V}{K}\right) = C^0(K V \; + V \; + V \;)$$

The mean relative tissue concentration after loss of the carryover solute is therefore

$$\frac{\bar{C}(t)}{C_0} = \frac{V}{V_c + \frac{V \; + V}{K}} \frac{\bar{C}\,(t)}{C^0} + \frac{V}{V_x + V_c K + V} \frac{\bar{C}_x(t)}{C^0} \qquad \{t > 0\}$$

where $\bar{C}\,(t)$ and $\bar{C}\,(t)$ are the mean cellular and extracellular concentrations at time t By algebraic manipulation this can be written

$$\frac{\bar{C}(t)}{C_0} = \frac{V K + V_x}{V K + V \; + V} \left[\frac{1}{1 + \frac{V}{V K}} \frac{\bar{C}\,(t)}{C_c^0} + \frac{1}{1 + \frac{V K}{V_x}} \frac{\bar{C}\,(t)}{C_x^0}\right] \qquad [t > 0] \quad (3)$$

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$$n_0 = C^0 \left(V \; + \frac{V_x + V}{K} \right) = C^0 (K V \; + V_x + V \;)$$

The mean relative tissue concentration after loss of the carryover solute is therefore

$$\frac{\bar{C}(t)}{C_0} = \frac{V}{V \; + \frac{V_x + V}{K}} \frac{\bar{C}\,(t)}{C^0} + \frac{V_x}{V \; + V \; K + V} \frac{\bar{C}\,(t)}{C_x^0} \qquad [t > 0]$$

where $\bar{C}\,(t)$ and $\bar{C}\,(t)$ are the mean cellular and extracellular concentrations at time t By algebraic manipulation this can be written

$$\frac{\bar{C}(t)}{C_0} = \frac{V \; K + V}{V \; K + V \; + V} \left[\frac{1}{1 + \frac{V}{V \; K}} \frac{\bar{C}\,(t)}{C^0} + \frac{1}{1 + \frac{V \; K}{V}} \frac{\bar{C}_x(t)}{C_x^0} \right] \qquad [t > 0] \quad (3)$$

so that the influence of carryover in the tissue kinetics is incorporated in the factor $(V_c k + V_x)/(V_c k + V_x + V\ )$

To evaluate this factor we determined the fractional carryover space $\alpha = V\ /(V_c + V_x + V\ )$ by weighing drained muscles before and after blotting and by dissecting and weighing the connective tissue pads Several determinations of carryover space gave 7 5–8 0 per cent of the total muscle mass the adherent fluid and connective tissue each contributing about 4 0 per cent

The First Approximation

A useful approximation is made possible by the fact that the cellular flux of many solutes is very slow in comparison to the extracellular flux In the limit the extracellular space empties essentially independently of the cellular space In this case extracellular flux is sufficiently rapid so that we may set $\bar{C}_x(t)/C_x^0$ equal to zero for all but a short initial time period The cellular flux is then given by Eqs 1 or 2 and $\bar{C}(t)/C_0$ can be evaluated from Eq 3 and the conditions

$$\bar{C}_x(t)/C_x^0 = \begin{matrix} 1 \text{ at } t = 0 \\ 0 \text{ for } t > 0 \end{matrix} \tag{4}$$

The Surface Permeation Model

The complete solution for the efflux kinetics from the sartorius in the surface permeation is given by Harris and Burn (1949) Taking the muscle to be a plane sheet of thickness B containing long parallel right circular cylinders of radius r_0 between which course extracellular channels of mean length λB they obtain the flux equations

$$\left.\begin{aligned} \frac{\partial C_c(t\ y)}{\partial t} &= -\sigma[C_c(t\ y) - kC_x(t\ y)] \\ \frac{\partial C_x(t\ y)}{\partial t} + \frac{V_c}{V_x}\frac{\partial C_c(t\ y)}{\partial t} &= \frac{D_x}{\lambda^2}\frac{\partial^2 C_x(t\ y)}{\partial y^2} \end{aligned}\right\} \tag{5}$$

where y is the diffusion coordinate $(0 \leq y \leq B)$ and D_x is the extracellular diffusion coefficient The starting and boundary conditions are

$$C\ (0\ y) = C^0 = kC_x(0\ y) = kC^0$$

$$C_x(t\ 0) = C_x(t\ B) = 0$$

for $t > 0$

The solutions averaged over y to give mean concentrations are

$$\bar{C}_t(t) = \frac{8C^0}{\pi^2}\sum_{k=0}^{\infty}\frac{1}{(2k+1)^2}\left[\frac{r_2 e^{r_1 t} - r_1 e^{r_2 t}}{r_2 - r_1}\right]$$

$$\bar{C}_e(t) = 8C_x^0\sum_{k=0}^{\infty}\left\{\frac{1}{(2k+1)^2\pi^2}\left[\frac{r_2 e^{r_1 t} - r_1 e^{r_2 t}}{r_2 - r_1}\right] + \frac{D_x}{l^2B^2}\left[\frac{e^{r_1 t} - e^{r_2 t}}{r_2 - r_1}\right]\right\} \quad (6)$$

where for each value of k, r_1 and r_2 are the roots of

$$r^2 + \left[\sigma\left(1 + \frac{V_e}{V_x}\lambda\right) + (2k+1)^2\pi^2 D_x/l^2B^2\right]r + \sigma(2k+1)^2\pi^2 D_x/l^2B^2 = 0$$

The tissue kinetics are given by these equations and Eq 3. Several curves plotted from these kinetics appear in Figs 3–5 labeled A. They have two important features: there is a short curved preexponential phase in contrast to the purely exponential kinetics for a single fiber, and the slope of the exponential phase[1] differs from σ, the cellular rate constant.

We may fix limits for the intercept I of the exponential phase on the ordinate axis for these kinetics. When the extracellular flux is relatively fast, the approximate analysis above gives a limiting intercept of $\mu/(1 + V_e/V_x\lambda)$ where μ is the carryover factor in Eq 3. When the extracellular flux is relatively slow, the kinetics approach diffusion from a plane sheet with intercept $8\mu/\pi^2$. Eqs 3 and 6 show these to be lower limits, i.e., the intercept is never less than the smaller of $\mu/(1 + V_e/V_c\lambda)$ and $8\mu/\pi^2$ and usually is larger than *either*. These limits are nearly equal numerically (see below) so that the minimum intercept is about 0.73.

The Bulk Diffusion Model

Because of mathematical difficulties analysis of the bulk diffusion model can be extended only semi-quantitatively from the first approximation. This will prove adequate for present purposes for two reasons: (1) The conditions for the validity of the first approximation will be shown to hold for the slowest moving solutes studied. (2) The range of intercepts appropriate to the bulk diffusion model can be specified with reasonable certainty and shown to differ from that for surface permeation.

[1] This phase does not in general correspond to a single exponential term in Eqs 6; usually there are two similar terms whose sum is linear to well within the precision necessary for this analysis.

The approximate analysis of bulk diffusion gives a minimum intercept of $\mu(4/\gamma_1)(1 + V_2/V_c A)$ since the intercept of Eq 1 must be included. This limit is about 0.53 whereas the minimum intercept in the surface permeation model was shown to be 0.73. Thus there is a range of intercepts 0.53 to 0.73 wherein the kinetics can only be bulk diffusion. Several curves plotted for bulk diffusion appear in Figs 3–5 labelled B.

It seems likely that $8\mu/\pi^2$ is an upper rather than secondary lower limit to the intercept in bulk diffusion, so that the intercept ranges in the two models are completely exclusive. This is because the physical origins of the intercepts in the two models differ essentially, one arises from a two compartment process and the other from a bulk diffusion. For a given cellular rate constant, as the extracellular diffusion becomes slower (i.e. as the first approximation is deviated from) the preexponential phase changes more rapidly than the exponential in the surface permeation model. There is reason to believe that the reverse occurs in bulk diffusion, because the extracellular concentration, while not falling instantaneously to zero as in the first approximation, still falls more rapidly than the average intracellular level, thus permitting the establishment of large concentration gradients in the cell, with the consequent long period of nonexponential flux which occurs before a quasi steady state is set up.

Comparison of Experimental and Theoretical Kinetics

Evaluation of Parameters

The clearest comparison of experimental and theoretical kinetics is provided by superposition of the curves in a single plot. We will use the complete expression of Eqs 3 and 6 for surface permeation kinetics and the approximate treatment for the bulk diffusion given by Eqs 1, 3 and 4. In this section we will discuss the choice of parameters for the theoretical curves and in the next section we will present the comparison of kinetics.

In the calculation of bulk diffusion curves for comparison with experiment, the value of the experimental limiting slope S of the exponential phase may be used directly for the theoretical slope $D_c\gamma_1/r_0$. The calculation of surface permeation curves is more difficult. The calculated limiting slope S is different from the cellular flux parameter σ and their relationship is not easily determined beforehand. We found it best to calculate curves using several values of σ in the range of the experimental slopes and then to choose for graphical

comparison curves of similar S_t and S The values used for σ were 2×10^{-4} 6×10^{-4} 10^{-3} 3×10^{-3} and 5×10^{-3} (sec^{-1})

$D\gamma_1^2/r_0^2$ and σ were the only parameters chosen by fit The others—V_e/V_s K D B λ—were chosen as follows

a *The extracellular space* V/V_s was taken equal to 7 corresponding to 12 5 per cent extracellular space We also calculated curves with V/V decreased to 4 giving an extracellular space of 20 per cent and found this had no important influence These values are all exclusive of the connective tissue pads at the ends of the muscle

The extracellular pace of close packed cylinders is 9 7 per cent compression of the cylinders will reduce even this One would expect the extracellular space of sartorius muscle to be near this value But reported values do not always confirm this they range from the widely accepted values of 8–13 per cent (Boyle *et al* 1941) and 1? 5 ± 0 4 per cent (Desmedt 1953) to 8–40 per cent (Tasker *et al* 1959) and 16–47 per cent (Johnson 1955) Most of the methods of determining extracellular space are based on the assumption that the probe molecule is excluded from the intracellular space while the measurement is made The wide range of reported values the great variability within a single study (Tasker *et al* 19 9) and the dependence on the physiological state or age of the tissue (Johnson 1955) make it doubtful that this assumption can be correct in any simple universal manner even for as large a molecule as serum albumin The assumption has never adequately been examined Generally its correctness is inferred when the equilibrium distribution of the probe molecule between the tissue and the bathing solution is less than unity This is hardly justified such distributions may indicate limited accumulation within the cells and are not inconsistent with rapid permeation (cf Bozler 1959 and the results for alcohols in the present work)

Since we are concerned here with the true anatomical extracellular space we have used values of V/V which seem most consistent with it

b *The partition coefficient* K of a solute between cellular and extracellular phases may be evaluated from the experimental data if it is assumed that the distribution coefficient between the extracellular space and free solution is unity and if a value of V/V is taken The values of K used to calculate theoretical curves 0 6 0 85 and 1 1 span the range of experimental values

Knowing the total tracer solute present in the muscle after incubation and the weight of the blotted muscle we may write for the weight based initial tissue concentration

$$C = \frac{n}{wt} = \frac{n}{\varrho(V\ + V_s + V\ )} = \frac{C_e^0 V\ + C_s^0(V\ + V\ )}{\varrho(V\ + V_s + V\ )}$$

The approximate analysis of bulk diffusion gives a minimum intercept of $\mu(4/\gamma_1)(1+V_x/V_c K)$ since the intercept of Eq 1 must be included This limit is about 0 53 whereas the minimum intercept in the surface permeation model was shown to be 0 73 Thus there is a range of intercepts 0 53 to 0 73 wherein the kinetics can only be bulk diffusion Several curves plotted for bulk diffusion appear in Figs 3–5 labelled B

It seems likely that $8\mu/\pi^2$ is an upper rather than secondary lower limit to the intercept in bulk diffusion so that the intercept ranges in the two models are completely exclusive This is because the physical origins of the intercepts in the two models differ essentially one arises from a two compartment process and the other from a bulk diffusion For a given cellular rate constant as the extracellular diffusion becomes slower (i e as the first approximation is deviated from) the preexponential phase changes more rapidly than the exponential in the surface permeation model There is reason to believe that the reverse occurs in bulk diffusion because the extracellular concentration while not falling instantaneously to zero as in the first approximation still falls more rapidly than the average intracellular level thus permitting the establishment of large concentration gradients in the cell with the consequent long period of nonexponential flux which occurs before a quasi steady state is set up

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c *The diffusion coefficient in the extracellular space* D_x was taken as 9×10^{-6} cm²/sec in all cases This is a modal value for the diffusion coefficients of the nonelectrolytes studied in aqueous solution

Several investigators have estimated D_x for compounds which enter tissues to a limited extent by assuming that part of the flux curve represents diffusion into the extracellular space These studies give values of D/λ^2B^2 where B is the actual tissue thickness and λ is the factor by which the diffusion path length exceeds B McLennan (1956 1957) studying mammalian muscle derived for λ the expression

$$\lambda = 1 + \frac{4}{\pi}\left(\frac{V_f}{V_m}\right)\left(1 - \frac{\pi}{4}\right)$$

where V_f/V_m is the fractional cellular space determined from the inulin space McLennan showed λ to be equal to 1 242 when V_f/V_m is 0 85 and found D_x for inulin and sucrose to be equal to or slightly greater than the diffusion coefficients in free solution D_w Johnson (195) studying frog sartorius muscle used a value of λ of $\pi/2$ and found D_x for sucrose to be 63 per cent of D_w Creese (1954) found D/λ for inulin in rat diaphragm to be 25 per cent of D_w McLennan (1957) using the data of Boyle *et al* (1941) for frog sartorius muscle found D of inulin to be 40 per cent larger than D_w

All of these studies show that the overall flux in the extracellular space as reflected in the measured apparent diffusion coefficient D/λ^2 is slower than in a comparable thickness of free solution It is not clear that this is attributable solely to increased path length rather than partly to decrease in the diffusion coefficient But for the present work this differentiation is unimportant since the factors occur only in the combined form D_x/λ^2B^2 so that λ may be incorporated into either D or B in general we choose the latter (see below) Since we have studied compounds whose molecular weights range from 32 to 104 and a temperature range of 0 to 30 C the diffusion coefficient in the extracellular space will vary from one experiment to another Ideally this variation should be reflected in the theoretical curves but this is neither necessary nor feasible for several reasons (1) it may be unwise to extrapolate too finely the conclusions based on studies of diffusion of the large molecules inulin and sucrose to the small molecules of the present study (2) the calculation of theoretical curves in the surface permeation model is so lengthy as to make detailed calculations for each experiment impossible (3) in the bulk diffusion approximation we can account only very roughly for extracellular diffusion (4) those aspects of the theoretical curves with which we are most concerned are quite insensitive to variations in D of the magnitude likely to occur

Combination of the assumed value of D with the different values of B mentioned below enable us to span the appropriate range of values of D/λ^2B^2

where V is the volume of the connective tissue pads representing about 4 per cent of the total tissue and ρ is the density of the tissue. Assuming that C_x^0 is equal to C_s the concentration of tracer in the incubation solution we derive for the distribution coefficient

$$K = \frac{C_c^0}{C_x^0} = \rho\frac{C_0}{C_s}\left(\frac{\alpha_c + \alpha_x + \alpha}{\alpha_c}\right) - \frac{\alpha_x + \alpha}{\alpha_c}$$

where $$\alpha_i = V_i/(V_c + V_x + V)$$

Taking $\rho = 1\ 06$ $\alpha/\alpha_x = 7$ $\alpha = 0\ 08$, $\alpha = 0\ 04$ we get

$$K = 1\ 265\ C_0/C_s - 0\ 242$$

The values of K found for the compounds studied are given in Table 3

TABLE 3 *The equilibrium distribution coefficient K between the cellular and extracellular spaces in R pipiens sartorius at low and high temperatures*

	Temperature (C)	K
methanol	1 5	0 74
	15 3	0 79
ethanol	1 6	0 70
	15 1	0 81
n propanol	1 5	0 73
	15 2	0 73
n butanol	7 4	0 89
	20 4	0 84
formamide	2 9	0 96
	19 6	0 92
acetamide	1 1	0 99
	20 9	0 87
propionamide	0 8	1 00
	20 8	0 97
thiourea	1 6	1 28
	20 5	1 08
methyl thiourea	0 8	1 14
	20 8	1 10
ethyl thiourea	0 8	0 90
	20 2	0 97

c *The diffusion coefficient in the extracellular space* D was taken as 9×10^{-6} cm / ec in all cases This is a modal value for the diffusion coefficients of the nonelectrolytes studied in aqueous solution

Several investigators have estimated D_x for compounds which enter tissues to a limited extent by assuming that part of the flux curve represents diffusion into the extracellular space These studies give values of $D/\lambda B$ where B is the actual tissue thickness and λ is the factor by which the diffusion path length exceeds B M Lennan (1956 1957) studying mammalian muscle derived for λ the expression

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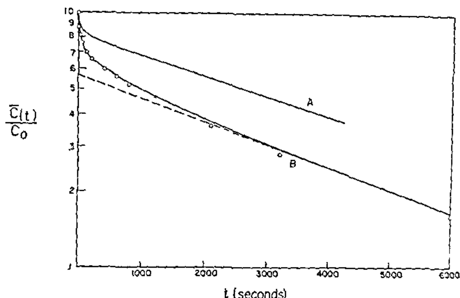


Fig 2 *Efflux of thiourea from the sartorius of R pipiens at 1 7 C* Ordinate mean fractional tissue concentration Points are experimental Curve A theoretical efflux based on surface limited cellular flux Curve B theoretical efflux curve based on bulk diffusion limited cellular flux

d *The thickness of the muscle B* was measured to be 0 05–0 065 cm As pointed out above it is desirable to modify this parameter in most instances to include the effect of increased diffusional path length by multiplying *B* by a factor whose value is in the range 1 20–1 25 We have chosen the values 0 055 and 0 075 for this analysis to include the ranges of both uncorrected and corrected path lengths

Comparison

a *Sartorius* In Figs 2 3 and 4 the experimental points in the efflux of thiourea at 1 7 C acetamide at 0 9 C and methanol at 7 5°C respectively are plotted with theoretical curves for surface permeation (labeled A) and bulk diffusion (labeled B)

The parameters used were I_c/I 7 K 1 1 for thiourea and acetamide 0 6 for methanol (values from Table 3 were used for the carryover factor since this can be incorporated after the main calculation) D_x 9×10 cm sec^{-1} λB 0 07 cm for thiourea and acetamide 0 0 5 cm for methanol (to balance the underestimation of D_x) σ 10 sec^{-1} (thiourea) 1×10 sec^{-1} (acetamide) $5 \times 10^{-}$ sec^{-1} (methanol) D/r_0^2 was fitted The effect of improving the values of σ K and D_x/λ^2B^2 is too small to influence the comparison

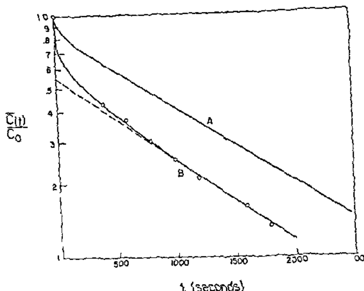


Fig 3 *Efflux of a tamide f om the sartorius of R pip ens at 0 9 C* Ordinate m an fra tional tissue concentration Points are experimental Curve A theoretical fflux based on surface li nited cellular flux Curve B theoretical efflux cur e based on bulk d ffusion limited cellular flux

Figs 2–4 show that the experimental efflux is entirely inconsistent with urface permeation kinetics but agrees well with the approximate bulk diffusion treatment The same was true for all of about sixty experiment

The detailed fit of experimental points to the bulk diffusion curves is be t for slow moving solutes such as thiourea compare Figs 2 and 4 Thi is expected from the approximation Nonetheless the fit is striking and unambiguous for all solutes

The agreement of the experimental efflux curves with a theoretical model based on the assumption of uniform resistance to transport throughout the entire cell is a serious objection to membrane per meability models However the basic feature of nonelectrolyte tran port which membrane permeation models attempt to explain is the rates of flux rather than the form of the flux curve The question ari e whether a model can be constructed combining membrane and bulk processes in such a way that the rate of flux is controlled by per meation while the form of the kinetics is determined by bulk diffusion

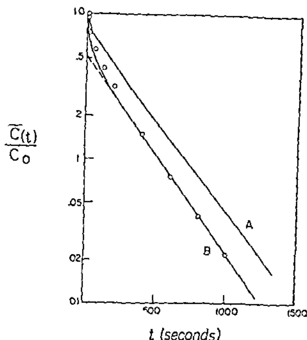
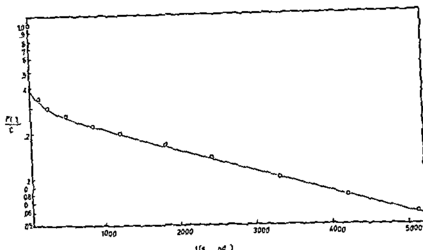


Fig 4 *Efflux of methanol from the sartorius of R pipiens at 7 4 C* Ordinate mean fractional tissue concentration Points are experimental Curve A theoretical efflux based on surface limited cellular flux Curve B theoretical efflux curve based on bulk diffusion limited cellular flux

This is impossible primarily because the large preexponential phase in diffusion kinetics is due to rapid unhindered flux under large concentration gradients at the surface A surface barrier will affect this phase most neutralizing the effect of the large gradients We can show this quantitatively for a single cylindrical fiber with resistive surface for which the theoretical relationships of intercept slope and rate constants can be obtained This demonstration is lengthy and has been deferred to Appendix I

b *Extensor longus digiti IV* The extraneous volume of the extensor preparation is large and variable Consequently theoretical curves based on an independently determined carryover will be unreliable Nevertheless the better exposure of fibers to bathing solutions in the extensor than in the sartorius makes the approximate bulk diffusion equations (Eqs 1 3 and 4) more appropriate in principle to this tissue A comparison of experimental flux in the extensor with these equations therefore provides a check on the conclusion drawn from the sartorius studies



F g 5 *Efflux of th ou ea from the extensor at 1 ° C* Po nts are experimental sol l curve is theoret al efflux based on bulk diffusion limited cellular flux

The efflux of thiourea from the extensor at 1 2 C is shown in Fig 5 The points are experimental and the line is a bulk diffusion curve This curve is plotted from Eqs 1 3 and 4 but there is an important difference between it and the sartorius curves the carryover in the extensor is determined by fitting the position of the diffu ion curve to that of the experimental points whereas in the sartorius the carryover is measured independently

The combined carryover and extracellular space from Fig 6 is 61 per cent which seems reasonable The initial sharp drop in con centration which thus represents is distinct from the long preexpone n tial phase which follows The latter lasts about 1000 seconds much longer than could be accounted for by diffusion *from adherent* connective tissue In Fig 6 the efflux of *n* butanol from the extensor at 1 3 C is presented The flux is more rapid than in *the sartorius* This curve shows a feature which appears in the extensor studies at low concentrations—a small (0 5 *to 3 per cent*) very slow fraction of uncertain origin In order to obtain the principal exponential phase of the kinetics for calculation *of* D_t the extrapolated contribution of the slow fraction was subtracted as shown in Fig 6

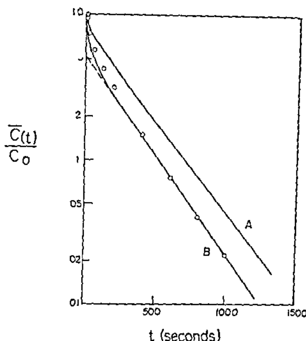


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CHAPTER 3

The Diffusional Parameters in Muscle Cytoplasm

In the preceding chapter it was shown that the efflux of the simple organic nonelectrolytes from frog muscle follows the kinetics of a cytoplasmic diffusion process with no evidence for the influence of a surface or membrane permeation process In this chapter the diffusion parameters which characterize the experimental kinetics will be evaluated They will be used in the following chapter as the basis for examining the hypothesized cytoplasmic diffusion in the light of diffusion in other physical systems and the properties of cytoplasm

Evaluation of the Diffusion Coefficient in Muscle Cytoplasm, D

Equations 1 3 and 4 give the following relationship between S and the diffusion coefficient in muscle cytoplasm D_c

$$S = D \gamma_1 / r_0^2$$

All diffusion coefficients will be calculated from this using the values of S given in Tables 1 and 2 The values obtained in the sartorius and the extensor will be distinguished by the symbols D_S and D_E respectively

The value of γ_1 is 5 83 The values for the mean cellular radius r in the two muscles were determined by measurement of cellular cross sectional areas in photomicrographs of the tissues fixed in Susa and imbedded in paraffin The radii were corrected for shrinkage in processing by assuming this to be proportional to the linear shrinkage undergone by the tissue The mean values of r determined were 34 9 μ and [illegible]4 4 μ for the sartorius and extensor respectively

A comparison of D_S and D_E is provided by Table 4 which gives values interpolated to 5 C and 20 C from the least squares Arrhenius lines described on p 28 and by Figs 7 and 9 It can be seen from this

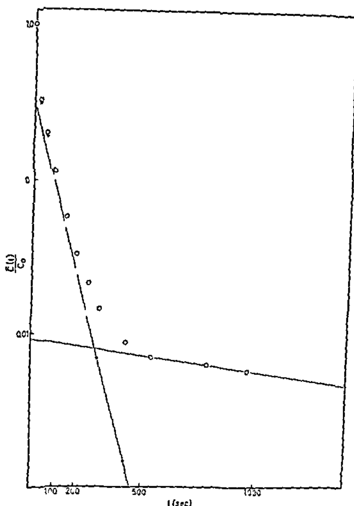


Fig 6 *Efflux of n butanol from the extensor at 1 3 C* Open circles are experimental points closed circles represent the residual flux after the slow component has been subtracted

The efflux of *n* butanol has a large preexponential phase like that of thiourea but we shall not attempt a precise delineation of it There is nothing new to be learned and the uncertainties already described are accentuated by the rapid flux

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Evaluation of the Diffusion Coefficient in Muscle Cytoplasm, D

Equations 1 3 and 4 give the following relationship between S_e and the diffusion coefficient in muscle cytoplasm D

$$S_e \quad D \gamma_1 / r_0^2$$

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TABLE 4 *Interpolated diffusion coefficients in the sartorius, D_S and extensor, D_E at two temperatures for three nonelectrolytes*

	$10^9 D_E$ (cm²/sec)		$10^9 D_S$ (cm²/sec)	
	5 0 C	20 0°C	5 0 C	20 0 C
n butanol	15	27	4 9	7 3
propionamide	6 8	15	2 8	4 4
thiourea	0 46	2 0	0 50	1 3

table that there is good agreement between D_S and D_E for the slow moving thiourea At any temperature the agreement becomes progressively poorer for the more rapidly diffusing compounds Also the agreement is better the lower the temperature These relationships are in accord with the expected effect discussed in Chapter 2 of extracellular diffusion and provide a notion of the range in which this effect becomes significant

Since there is a substantial difference between D_S and D_E for most compounds principal reliance in the remainder of this work will be placed on D_E

Temperature Dependence

In Fig 7 ln D_E is plotted against $1/T$ for thiourea propionamide and *n* butanol The linear relationships permit the temperature dependence to be described by Arrhenius equations

$$D_E = D_E^0 e^{-E/RT}$$

where D_E^0 is a temperature independent frequency factor and E an activation energy The values of these parameters are given in Table 5

TABLE 5 *Arrhenius parameters for D_E for three nonelectrolytes in the extensor*

	D_E^0 (cm²/sec)	E (kcal/mole)
n butanol	1 8 10^{-2}	6 4
propionamide	3 [illegible] 10^{-2}	8 6
thiourea	8 3 10^{2}	15 6

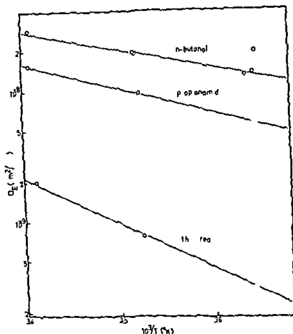
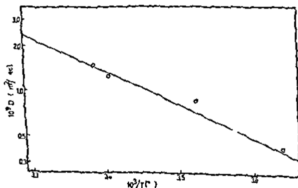


Fig 7 *Arrhen us plot of D_E for th ourea propionamide and n butanol*

In the sartorius a non linear relationship may be expected because two processes—extracellular and cytoplasmic diffusion—make significant contributions to the flux However the precision of available data does not permit resolution of this non linearity It is found that



F g 8 *Arrhenius plot of D_S for thiourea*

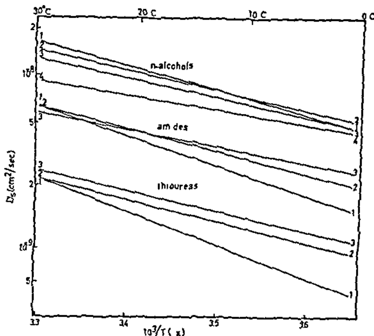


Fig 9 *Least squares Arrhenius lines for Ds for ten compounds* The *n* alcohols are (1) methanol (2) ethanol (3) *n* propanol (4) *n* butanol the amides are (1) formamide (2) acetamide (3) propionamide the thioureas are (1) thiourea (2) methyl thiourea (3) ethyl thiourea

a least squares Arrhenius line adequately represents the temperature dependence of D_s as shown in Fig 8 for thiourea The least squares lines for D_s for ten compounds are presented in Fig 9 Within a group of homologous compounds the slopes of these lines are distinct Although the probable error in ordinate position of the lines is large the separations thiourea–ethyl thiourea and formamide–propionamide are significant at low temperatures Because of the impossibility of assigning the observed Arrhenius parameters for D_s to either of the two processes involved in the flux these parameters will not be presented

Chemical Specificity

The pattern of diffusional specificity in respect to chemical grouping is seen in Figs 7 and 9 The nature of the polar grouping—alcoholic amide or thiourea—dominates the diffusion coefficients without regard to molecular size Among compounds of equal molecular size the most polar diffuses most slowly Since diffusion in the extracellular

space reduces the extent of observable chemical specificity in the sartorius a better estimate of the magnitude of specificity is obtainable from the extensor The three compounds in Fig 7 are of nearly equal molecular weight the spread in D_E between thiourea and n butanol is fifty fold at 0 C and fifteen fold at 20 C This is comparable to the spread in Chara (Collander and Barlund 1933)

The data of Table 5 show the chemical specificity in D_E to be energy controlled as is usual in diffusional processes

Dimensional Specificity—the influence of alkyl groups

It can be seen in Fig 9 that the pattern of specificity in D_S among the members of each homologous class is temperature dependent Two processes determine these patterns cellular and extracellular diffusion

These patterns can be clarified somewhat by a recognition of the probable contribution of extracellular diffusion The latter may be expected to resemble diffusion in free aqueous solution in showing a small temperature dependence (corresponding to an activation energy of about 5 kcal) and a pattern in which larger molecules in a homologous series diffuse more slowly than smaller Since this temperature dependence is smaller than that of most of the lines in Fig 9 the relative contribution of extracellular diffusion will be greatest at high temperatures and least at low where the most reliable estimate of dimensional specificity in cellular diffusion in the sartorius is obtainable Among the thioureas and the amides the largest members diffuse most rapidly at low temperatures The overall separation in each group about two fold near 0 C represents a minimum estimate of the true cellular specificity The reduction in spread among the thioureas at high temperatures and the crossover among the amides leading to an inverted order in D_S are favored by the increased contribution from extracellular diffusion in this range However if the low temperature specificity pattern is energy controlled as the data suggests the cellular diffusion itself will show the same tendency toward inversion of order at high temperatures Thus the high temperature tendency may have a dual origin and the relative contributions are not specifiable from present data

Dimensional specificity among the alcohols in the full temperature range resembles that in the amides at high temperatures The cellular

flux of alcohols is very rapid so that extracellular diffusion is likely to contribute significantly to the order observed This order is consistent with a variety of patterns of cellular flux to resolve these and the uncertainties in the amide and thiourea data additional studies using the extensor will be necessary

CHAPTER 4

Origins of the Diffusional Properties of Cytoplasm

The presumption that nonelectrolyte transport in cells is a bulk diffusional process draws our attention to both the composition and molecular organization of cytoplasm The gross composition of cyto plasm as a protein water-ion system is relatively well known and must be considered as a baseline in characterizing the system as a diffusional medium Yet few physical properties of such systems can be defined by composition alone and much evidence exists for believ ing that the particular organization of the cytoplasmic components is responsible for the unique characteristics of this material Of special interest to the understanding of nonelectrolyte transport is the evid ence that significant organization extends to the level of cytoplasmic water The importance of this may be understood when one considers that cytoplasm is about 80 per cent water so that the molecular neigh bors of a diffusing solute are almost entirely water molecules

The Properties of Cytoplasmic Water

The principal determinants of the interactions of a water molecule are its dipole moment of 1 87 debyes and its ability to form four tetra hedrally directed hydrogen bonds The energy of the latter depends upon both the electronic properties of the second molecular species and the geometry of the bond The maximum bond energy is realized when a linear nuclear array is possible The effect of nonlinearity is to reduce the bond energy roughly according to a cosine law for small angles and more rapidly for large The energy of an unrestrained hydrogen bond between two water molecules is about 5 kcal/mole (Coulson 1959 Pimentel and McClellan 1960)

The crystalline structure of ice is determined by the tetrahedral orientation of the hydrogen bonding directions of the water molecule Each molecule is linearly bonded to four nearest neighbors a loose packing which results in an open lattice The breaking or bending of

TABLE 6 *Depth of the water layer stabilized by a variety of materials*

Material	Depth (Å)	Method
Mica[a]	1000–2000	compressive strength
Mica[b]	2000–5000	dielectric constant and dispersion
Montmorillonite[c]	10–20	x ray diffraction
Silica and Glass[d]	45–5300	multilayer adsorption
Lipid emulsion[e]	85	x ray diffraction
Soap micelle[f]	42	x ray diffraction
Desoxyribonucleic acid and hyaluronic acid[g]	500–1000	streaming dielectric
Crystalline Methemoglobin[h]	15–25	x ray diffraction
Turnip yellow mosaic virus[i]	78	x ray diffraction

Reference

a DERJAGUIN and KUSSAKOV 1939 *b* PALMER CUNLIFFE and HOUGH 1952 *c* HOFFMANN and BILKE 1936 SHAW 1942 *d* LENHER 1924 MCHAFFIE and LENHER 1925 *e* SCHMITT and PALMER 1940 *f* HESS *PHILIPPOFF and KIESSIG 1939 g JACOBSON 1955b JACOBSON* and LAURENT 1954 *h* BOYES WATSON DAVIDSON and PERUTZ 1947 *i* BERNAL and CARLISLE 1948

hydrogen bonds associated with melting relaxes the spatial requirements of the lattice and permits closer molecular packing However water cannot be treated as a structureless close packed liquid Much evidence exists that like ice the liquid shows substantial lattice ordering While the structural forms involved are still subject to investigation agreement is universal that water molecules exist in a variety of more or less extensive quasi crystalline orderings These are based as in ice on the four tetrahedrally directed hydrogen bonds but they may in any region have only transient existence longer at lower temperatures shorter at higher temperatures (Bernal and Fowler 1933 Forslind 1952 Frank 1958 Pauling 1935 Pople 1951)

The stability form and extent of the lattice ordering is profoundly influenced by the presence of solutes The solutes that principally determine the properties of cytoplasm are protein This follows from the high concentrations of protein in cells and the fact that all of the functional groups of proteins are capable of interacting with water

These functional groupings fall broadly into three classes ionic hydrogen bonding and nonpolar Some notion of the interactions of proteins may be inferred from the aqueous properties of monomeric solutes containing these functional groupings—such as the hydration of ions the generation of low entropy structured domains by nonpolar molecules and the hydrogen bond interactions between solvent and solute in aqueous solutions (Claussen 1951 Feates and Ives 1956 Frank and Evans 1945 Robinson 1946) As significant as these interactions are much evidence suggests that even more extensive interaction of protein with water is made possible by the stabilization of arrays of functional groupings by the covalent linkages of the polypeptide backbone (Jacobson 1955a Ling 1962 Chap 2)

Two variables in this fixed array seem important the nature and reactivity of the interacting sites and their spatial distribution When these are appropriate the macromolecule may impose or stabilize an ordered water lattice whose extent is greater than and whose fluctuations are smaller than those of liquid water at the same temperature (Jacobson 1955a Klotz 1958) Other conditions which have been suggested as favoring extensive structuring include the presence of elongated fibrous proteins rather than globular ones the matching of structured domains denseness and regular packing of macromolecules Each of these conditions appears to be fulfilled by the proteins in muscle

The distances from a matrix over which the stabilization of water structure may be enhanced can be large Estimates taken from various literature references are given in Table 6

The distance that separates adjoining protein backbones in protoplasm can be shown to average 16 9 Å (Ling 1962 Chap 3) the magnitudes presented in Table 6 make plausible the possibility that all of the water interspersed between cellular protein molecules is highly ordered Of possible importance also is the fact that cellular proteins are probably laid down in particular patterns in the process of synthesis if these patterns are such that the lattice ordered domains of individual protein fibers are able to match a great extension of lattice stabilization could come about The observation that the crystallinity of pectinic acids (Palmer Merrill and Ballantyne 1948) and collagen (Harrington and Von Hippel 1961) is enhanced by increased water content may be relevant to a reciprocal role of the water lattice on the pattern of protein laydown (Jacobson 1955a)

Evidence for structuring of water has been elicited in direct studies in cells using the phosphorescence of dyes (Szent Gyorgyi 1957) and nuclear magnetic resonance (Odeblad 1959 1960) Physiological processes in which ordered water lattices have been suggested to play a role are muscle contraction and photosynthesis (Szent Gyorgyi 1957) ionic selectivity (Baird *et al* 1957 Ling 1962 Chap 4) energy and electron transport (Klotz 1962) and signal propagation and radiation damage (Privalov 1958)

The above discussion makes it clear that in examining the transport properties of cytoplasm as an aqueous system one must be prepared to consider the water as modified from its pure state This modification seems to be in the direction of increased lattice ordering While the details of the structure of protoplasmic water are unknown some general properties can be assumed These are that protoplasmic water in comparison with pure water has reduced translational and rotational freedom and a statistically more orderly microscopic structure In these respects the properties of the water of cytoplasm may be thought of as intermediate between those in liquid water and in crystalline aqueous systems such as ice and crystal hydrates Therefore examination of the influence of these microscopic properties on the diffusional behavior of water ice and related physical systems should provide a basis for assessing the physical plausibility of the bulk diffusion model of nonelectrolyte transport

The Magnitude of the Diffusion Coefficients

The self diffusion coefficient in liquid water at 0 C is about 10^{-5} cm^2/sec while that of ice is about 10^{-10}cm^2/sec (Kuhn and Thurkauf 1958 Wang Robinson and Edelman 1953) This type of discontinuity in the magnitude of D is not uncommonly associated with phase changes It parallels the microscopic phenomena associated with the increased crystallinity itself These are in the case of water a decrease in translational and rotational freedom consequent on an increase in the strength and frequency of bonds and a reduction in fluctuation of orientation of molecules about the linear hydrogen bonding direction a reduction in the number of lattice defects and the requirement of cooperative involvement of a number of hydrogen bonds in the neighborhood of a structural distortion The preceding discussion has indicated that these features are common to ice and cytoplasmic water

Insofar as such concomitants of lattice ordering determine the diffusion coefficient in aqueous systems one may expect that cytoplasmic water will present a greater barrier to diffusion than the pure liquid The diffusion coefficients calculated for water in a variety of cells by Dick (1959) fall in the range $1\ 5 \times 10^{-8}$ to $5\ 0 \times 10^{-10}$ cm²/sec and those calculated for a variety of nonelectrolytes in the present study 3×10^{-8} to 3×10^{-10} cm²/sec These values do fall between those of ice and water and in a loose sense agree with expectation However it is desirable to obtain independent estimates of the appropriateness of these values

The evidence for lattice ordering presented above is largely concerned with the equilibrium extent of lattice ordering and gives no direct information of the dynamical properties of the lattice from which an independent estimate of the diffusional barrier could be inferred In order to obtain such an estimate it is necessary to turn to measurements of rate processes in cytoplasm and other aqueous systems These are basically of two types wave propagation processes of which dielectric dispersion has been most studied and transport processes of which next to diffusion itself viscosity is most important Unfortunately the interpretation of viscosity and dielectric dispersion in cells is made difficult by the complexity of the material The presence of macromolecules and other solutes gross structural heterogeneity and the metastability of the system may all make significant and often unresolvable contributions Nevertheless we believe that if cognizance is taken of these uncertainties these processes may be examined with profit

Evidence from Dielectric Dispersion Measurements

Near the melting point liquid water has a dielectric dispersion of about 9×10^{9} cycles/sec and ice of about 8×10^{3} cycles/sec A number of mechanisms have been suggested to explain these values involving proton displacements rotation of water molecules or the bending of hydrogen bonds (Forslind 1952 Haggis Hasted and Buchanan 1952 Pople 1951) Regardless of the particular mechanism it seems likely that the unit dielectric process involves a molecular movement or hydrogen bond breakage which is closely related to the unit process in diffusion (Frank and Wen 1957 Wang Robinson and Edelman 1953) This relationship may be roughly formalized by equating the characteristic time τ in the equation

$$D = \frac{\lambda^2}{\tau} \tag{7}$$

with the dielectric relaxation time defined as $1/2\pi f$ where f is the dispersion frequency Taking the unit displacement λ as 1 5 Å (Glasstone Laidler and Eyring 1941 Wang Robinson and Edelman 1953) and the dispersion frequencies given above we obtain diffusion coefficients of 10^{-5} and 10^{-11} cm²/sec for water and ice respectively, in rough agreement with the experimental values

Protein solutions show a characteristic dispersion at 10^5–10^7 cycles/sec (Oncley 1943) While the interpretation of this is still subject to investigation evidence has been presented that the observed dispersion is attributable to lattice ordered water in the vicinity of the macromolecule (Jacobson 1955*b*) [1] These dispersion frequencies would be expected according to Eq 7, to give diffusion coefficients in the lattice ordered water of 10^{-8} to 10^{-10} cm /sec

In protein solutions the ordered lattices do not form a continuum and therefore the gross diffusion coefficients for such systems only partially reflect the ordered water being determined largely by the free solution If as hypothesized the lattice ordering of cytoplasm were essentially continuous the observed cytoplasmic diffusion coefficients would be those calculated for the lattice Values for D_E obtained in the present study and for water by Dick (1959) agree in magnitude with the latter

Evidence from Viscosity Measurements

The Stokes Einstein equation

$$D = kT/6\pi\eta r \tag{8}$$

where r is the radius of the diffusing particle and η the gross viscosity of the medium *although developed for the diffusion of colloidal particles*, approximately describes the covariance *of diffusion and viscosity for small molecules in simple hydrogen bonded systems of widely* varying viscosity (Irani and Adamson 1958 Marinin 1956) This is shown by Fig 10 in which D is seen to vary as an inverse function

[1] Three dispersion regions have been observed in muscle tissue (Schwan 1957) One of these the β dispersion corresponds in frequency range to that seen in protein solutions However the interpretation of this is complicated by the possibility of gross structural relaxation (Maxwell Wagner mechanism) as well as by uncertain knowledge of molecular relaxation mechanisms

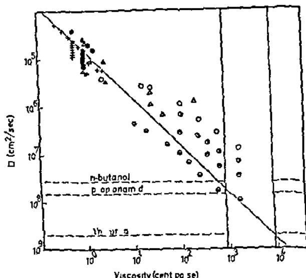


Fig 10 *D ffusion coefficient as function of visco ity in H bonding sy tems* The sol d line is plotted from the equat on

$$D = kT/6\pi\eta r$$

for -1 5 Å

The sy tems represented are + mutual or self diffus on of nonelectrolytes in o gan c solvents (Int Crit Tables V Johnson and Babb 1956) ● mutual d ffu on of nonelectrolytes in water (Int Crit Tables V Johnson and Babb 1956) and self diffusion of water at various temperatures (Wang, Rob nson and Edelma 1953) △ self diffusion of water and sucrose in aqueous sucrose solu ti n (Irani and Adamson 1958) ○ mutual diffusion of water ◐ methanol acetan le and sucrose n glycerol–water and glycerol-alcohol mixtur s (Mari un 1955 19 f)

The overlay represents Seifriz (1936 Ch 13) estimate of the usual rang of cytopla m iscosity The dott d l nes are the values of D_f at 20 C

of η through a fourfold range of magnitudes The reason for the success of this relationship lies in the similarity of the unit processes in diffusion and viscosity (Glasstone Laidler and Eyring 1941) in such simple systems

Cross vi cosity measurements are available for cytoplasm but in systems containing macromolecules several kinds of unit processes both structural and molecular may be operative in determining visco ity

Structural Components of Viscosity Structural interactions have little influence on small molecule diffusion and when they dominate

$$D = \frac{\lambda^2}{\tau} \quad (7)$$

with the dielectric relaxation time defined as $1/2\pi f$ where f is the dispersion frequency. Taking the unit displacement λ as 1.5 Å (Glasstone, Laidler and Eyring, 1941; Wang, Robinson and Edelman, 1953) and the dispersion frequencies given above, we obtain diffusion coefficients of 10^{-5} and 10^{-11} cm²/sec for water and ice, respectively, in rough agreement with the experimental values.

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In protein solutions the ordered lattices do not form a continuum and, therefore, the gross diffusion coefficients for such systems only partially reflect the ordered water, being determined largely by the free solution. If, as hypothesized, the lattice ordering of cytoplasm were essentially continuous, the observed cytoplasmic diffusion coefficients would be those calculated for the lattice. Values for D_E obtained in the present study and for water by Dick (1959) agree in magnitude with the latter.

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TABLE 7 *Correlation of S_e in the extensor and K_w^**

	Temperature (C)	$S \times 10^3$ (sec⁻¹)	K_w ether/water
n butanol	10 9	18 4	7 7
propionamide	10 5	9 03	0 013
thiourea	10 4	0 745	0 0063

Values for K_w were taken from Collander (1949)

ponent for the purpose of predicting diffusion coefficients must recognize two complicating factors structural viscosity arising from the cellular proteins and unrelated to diffusion and the non Newtonian nature of cellular viscosity These cannot be quantitatively assessed but their influence on the estimation of diffusion coefficients from gross viscosity will be in opposite directions

With the assumption that a major portion of cellular viscosity is molecular in origin a range of diffusion coefficients may be predicted using Eq 8 and the values of cellular viscosity given by Seifriz (1936 Chap 13)[1] The coincidence of predicted values and the values of D_E obtained in the present study is shown in Fig 10

Chemical Specificity

The flux rates of nonelectrolytes are markedly influenced by the nature of functional groupings in the diffusing molecule In Chapter 3 the pattern and extent of this chemical specificity are described The flux rates show a positive correlation with the equilibrium distribution coefficients K_w between a nonpolar solvent such as ether and the polar solvent water as shown in Table 7 In the classical membrane hypothesis this correlation is attributed to the participation in the transport of a distribution process between a nonpolar membrane and aqueous solution Such a process is precluded in an hypothesis like the present one in which nonelectrolyte transport properties are attributed to bulk diffusion in cytoplasm chemical specificity must arise from the diffusional process itself In this hypothesis the covariance of K_w and D signifies not the actual involvement of a distribution

[1] See Append x II fo th ju tifi ation for the use of these values The va ia t n n D_E among the three compound wh ch s out de the *domain of Eq 8* s xam ned in the section n c he n cal specificity

they lead to the dissociation of observed gross viscosity and diffusion This is exemplified by the study of Wang Anfinson and Polestra (1954) Concentrated (24 per cent) solutions of ovalbumin have a viscosity six times that of water and the self diffusion coefficient of ovalbumin is one sixth that extrapolated to infinite dilution whereas the self diffusion coefficient of the water is 60 per cent of that *in pure* water In the case of proteins which form a network the dissociation of *apparent viscosity and small molecule diffusion* may *be* even more marked Friedman and Kraemer (1930) studied the entry of urea *from 3* per cent aqueous solutions into gelatin gels of 4 0 to 29 0 per cent protein While the consistency of such gels may be comparable to viscosities of 10^6–10^9 centipoise the diffusion coefficients are 30 to 50 per cent of those of water

Systems showing such dissociation characteristically contain large amounts of relatively free water While the individual protein molecules may be hydrated the ordered system is far from continuous Thus the relative displacement *of two macromolecules involves only* a minimal modification of the interspersed water Other mechanisms are more important *the intertwining of the polymer chains and the* energy dissipation in aligning macromolecules in the direction of shear (Alexander and Johnson 1950 Chap 13) Neither *of these* appreciably affects the diffusion of solutes in the nonhydrate water and thus relatively high diffusion coefficients may be observed even when gross viscosity is high

Molecular Components of Viscosity The second important influence *of a macromolecule on the* viscosity of a system arises from its ability to modify the molecular transport properties of the solvent itself (*Frisch and Simha 1956*) *The argument for the importance* of this mechanism for biological macromolecules has been presented by Jacob on (1955*a*) Viewed simply *the high viscosities of the materials* arise from the distortion of water lattices associated with the macromolecules as they move relative to each other The energy dissipated in this distortion and reflected in the observed viscosity provides a direct measure of the dynamical properties of water molecules in the lattice and this component of viscosity may *therefore be* expected to show a Stokes Einstein correlation to diffusion

It *is a* corollary of the hypothesized lattice ordering of cellular water that the molecular component of viscosity will make a large contribution to the *gross* viscosity The attempt to isolate this com

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[1] See Appendix II for the justification for the use of these values The variation in D_E among the three compounds which is outside the domain of Eq 8 is examined in the section on chemical specificity

TABLE 8 *Diffusion coefficients, D in alcohols and K_w^n in ether/water and isobutanol/water*

Compound	MW	$10^5 D$ (cm²/sec)	K_w^n ether	K_w^n isobutanol
in methanol at 15 C (Thovert from Int Crit Tables V)				
allyl alcohol	58.1	1.80	—	2.34
acetamide	*59.1*	*1.50*	0.0025	0.33
urea	60.1	1.24	0.00047	0.13
ethyl ether †	74.1	2.00	10	—
pyridine	79.0	1.58	1.2	—
propylene glycol	76.0	1.24	0.018	—
isopentanol	88.1	1.34	19	—
aniline	93.1	1.49	14	—
ethyl acetate †	88.1	2.10	8.5	7.2
urethane	89.1	1.41	0.64	1.78
glycerol	92.1	1.15	0.00066	0.10
in ethanol at 20 C (Öholm from Int Crit Tables V)				
1,1-diethoxyethane †	118.2	1.13	>1	
monoacetin	134	0.51	0.041	
in 96% ethanol at 20 C (Öholm from Int Crit Tables V)				
camphor †	152.2	0.66	>1	
glycerol	*92.0*	*0.41*	*0.00066*	

Within each group the compounds are listed in descending order of K_w^n. The compounds noted by † are unable to donate protons for H bonding and show inordinately large D in relationship to both molecular weight and K_w^n.

K_w^n in ether/water are taken from Collander (1949), those in isobutanol/water from Collander (1950) and work of the present authors. K_w^n for 1,1-diethoxyethane and camphor were estimated.

process in the transport but a common determinant in the unit processes underlying both diffusion and distribution.

The common determinant is readily adduced. The energetically dominant element in both is hydrogen bonding interaction. The chemical specificity of K_w^n arises largely from the relative number and strength of H bonds between the solute and the two solvents of greatly disequal H bonding ability. A parallel involvement of H

bonds exists in diffusion This is because the loosening of solute solvent bonds in the activation process reflects in the same manner as K_w the number and strength of these bonds

The Occurrence of Chemical Specificity in H bonding Liquids

There is nothing in the above mechanism that suggests its uniqueness to cytoplasm We may expect it to occur in other H bonding systems and to be reflected in a similar pattern of chemical specificity That this is the case may be seen from Table 8 in which the diffusion coefficients in methanol and ethanol for several substances separated into groups by molecular weight are given along with the appropriate ether/water and isobutanol/water distribution coefficients[1]

The existence of K_w correlated diffusion in liquids supports the idea that the correlation observed in cellular transport arises from the specificity of diffusion in cytoplasm Nevertheless one must expect marked differences in the magnitude of specificity between diffusion in liquids and in a stabilized system like cytoplasm For while specificity is common in diffusion it is never great in liquids while it is generally of notable extent in more crystalline systems (Jost 1952 Chs 4 5 and 9) Comparison of Tables 7 and 8 shows that consistent with this expectation a greater specificity is observed in cell than in the liquids A closer examination of the basis of the low specificity of diffusion in liquids can provide a better understanding of the greater specificity attributed to cytoplasmic diffusion

The Magnitude of Chemical Specificity in H bonding Liquids

The relation of chemical specificity to solute–solvent interaction advanced above suggests that the difference in the extent of specificity between D in liquids and K_w is attributable to a difference in the mode of involvement of solute solvent bonds in the two processes In the dissolution process the transfer of a solute molecule from the polar to the nonpolar phase involves the breakage of H bonds which to a first approximation is of a completeness comparable to that occurring in vaporization On the other hand the energy requirements for diffusion in liquids are less stringent involving only a fraction of the energy of vaporization (Glasstone Laidler and Eyring 1941 Ch

[1] In effect this observation constitutes a generalization of the Overton Collander Rule enunciated for cellular transport to include diffusion in other H bonding systems

TABLE 9 *Diffusion coefficients, D in water at 15°C and K_w^n in ether/water and isobutanol/water*

Compound	MW	$10^5 D$ (cm^2/sec)	K_w^n ether	K_w^n i obutanol
n propanol	60 1	0 87	1 9	5 54
allyl alcohol	*58 1*	0 90	—	*2 74*
acetamide	59 1	0 96	0 0025	0 33
urea	*60 1*	*0 94*	*0 00047*	*0 13*
isopentanol	88 1	0 69	19	—
urethane	89 1	0 80	*0 64*	*1 79*

Within each group the compounds are listed in descending order of K_w^n

Sources of K_w^n are the same as in Table 8 D is taken from Thovert as quoted in the Int Crit Tables V

9) This is reflected in the great uniformity of diffusion coefficients among liquids of widely varying composition all being near 10^{-5}cm / sec (Johnson and Babb 1956 Jost 1952 Ch 9) as well as in the low specificity in the alcohols in Table 8 and in water in Table 9 and Fig 11 Solids in general show a smaller reduction in the energy of diffusion in relation to the energy of sublimation and for molecular crystals there is evidence of a close correspondence of the two energies (Glasstone Laidler and Eyring 1941 Ch 9 Jost 1952 Ch 2 and 5) As an example the characteristic activation energy for transport process in ice is 12–13 kcal/mole (Auty and Cole 1952 Bradley 1957 Pryslov 1958) while the energy of sublimation is 11 7 kcal/mole On the other hand liquid water with an energy of vaporization of about 10 2 kcal/mole has a characteristic activation energy of 4 6 kcal/mole (Wang Robinson and Edelman 1953) The rupture of intermolecular bonds in liquid diffusion can therefore be viewed as being facilitated by the greater orientational freedom enjoyed by the molecules in the liquid state In H bonding liquids the facilitation may be visualized as arising because orientational freedom is great enough to permit the approach of H bonded groups the subsequent transfer of a bond from one group to another occurring with only small bond distortion Thus the contribution to the diffusional barrier of hydrogen bonds in polar liquids is small as will also be diffusional specificity based on these bonds

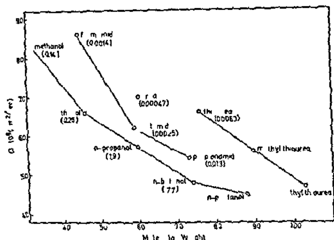


Fig 11 *Diffusion coefficients of 1% nonelectrolytes in a Sephadex^R–water gel as a function of molecular weight* The lines join members of homologous series The numbers in parentheses are the K_w^* in ether/ water (Collander 1949)

Chemical Specificity in Diffusion in Water

Before considering diffusion in cytoplasm in the context of H bonding specificity it is necessary to examine the special attributes of diffusional specificity in liquid water The specificity pattern observed in the alcohols is not seen in liquid water Rather as the data in Table 9 indicate the inverse pattern with respect to K_w^* seems to prevail A more complete picture of this inverted pattern than can be had from the limited data in pure water is shown in Fig 11 in which the diffusion coefficients for 12 nonelectrolytes in a Sephadex^R–water gel[1] are presented as a function of solute molecular weight (unpublished results of authors) The correlation of D for a given molecular weight with the values of K_w (given in parenthesis) can be seen to be the inverse of that in the alcohols

In view of the great potential for solute solvent H bonding in water one might expect a pattern of specificity comparable to that in the other H bonding liquids That water is anomalous in this respect is not surprising in view of the unusual properties of other water–nonelectrolyte interactions On the basis of the inordinately large

[1] Sephadex^R is the trade mark of AB Pharmacia Uppsala for cross-linked dextran In these experiments it was used as a gel in bead form containing about 80 water by weight

TABLE 9 *Diffusion coefficients D in water at 15°C and K_w^n in ether/water and isobutanol/water*

Compound	MW	$10^5 D$ (cm^2/sec)	K_w^n ether	K_w^n isobutanol
n propanol	60.1	0.87	1.9	5.54
allyl alcohol	58.1	0.90	—	2.74
acetamide	59.1	0.96	0.0025	0.33
urea	60.1	0.94	0.00047	0.13
isopentanol	88.1	0.69	19	—
urethane	89.1	0.80	0.64	1.78

Within each group the compounds are listed in descending order of K_w^n

Sources of K_w^n are the same as in Table 8. *D* is taken from Thovert as quoted in the Int. Crit. Tables V.

9). This is reflected in the great uniformity of diffusion coefficients among liquids of widely varying composition, all being near $10^{-5} cm^2/sec$ (Johnson and Babb, 1956; Jost, 1952, Ch. 9), as well as in the low specificity in the alcohols in Table 8 and in water in Table 9 and Fig. 11. Solids in general show a smaller reduction in the energy of diffusion in relation to the energy of sublimation, and for molecular crystals there is evidence of a close correspondence of the two energies (Glasstone, Laidler and Eyring, 1941, Ch. 9; Jost, 1952, Ch. 2 and 5). As an example, the energy of activation for transport processes in ice is 12–13 kcal/mole (Kuhn and Cole, 1952; Bradley, 1957; Privalov, 1958), while the energy of sublimation is 11.7 kcal/mole. On the other hand, liquid water, with an energy of vaporization of about 10.2 kcal/mole, has a characteristic activation energy of 4.6 kcal/mole (Wang, Robinson and Edelman, 1953). The rupture of intermolecular bonds in liquid diffusion can therefore be viewed as being facilitated by the greater orientational freedom enjoyed by the molecules in the liquid state. In H bonding liquids the facilitation may be visualized as arising because orientational freedom is great enough to permit the approach of H bonded groups, the subsequent transfer of a bond from one group to another occurring with only small bond distortion. Thus the contribution to the diffusional barrier of hydrogen bonds in polar liquids is small, as will also be diffusional specificity based on these bonds.

is comparable to that in hole formation The total energy $E_0 + E_1$ which may be viewed as an upper limit to the activation energy for diffusion of water in aqueous systems will thus be about 20 kcal/mole In the diffusion of other nonelectrolytes E_1 will depend upon the strength and number of H bonds between the solute and the water molecules of the lattice and chemical specificity will arise from this variation in E_1 a process comparable to but more selective than that which occurs in H bonding liquids

In the real systems involved in the present study the lattice structuring is far from perfectly rigid so that one cannot expect the simple rigid model to be entirely applicable In non rigid models the separation of two components in the unit diffusional process is less clear cut Furthermore an effect of the solute on the local state of the lattice becomes admissible The result of this in terms of the above model is to make E_0 as well as E_1 dependent on the nature of the solute because E_0 is determined by the state of the lattice in the vicinity of the solute The stability of the ordered array of water in cytoplasm is dependent upon the H bond interaction of the individual water molecules as well as the stabilizing influence of the macromolecules When a solute replaces one or more water molecules in the lattice its influence will be to either strengthen or weaken the lattice structure depending upon its size and the strength and orientation of its H bonding groups Solutes having multiple H bond directions and the possibility of orientating to fit the lattice can be expected to have a minimal disordering effect and may even strengthen the lattice Of the solutes employed in the present study this description best fits the smaller members of the more polar series—the thioureas and amides—which have the highest measured activation energies

When a solute is relatively nonpolar there will be disordering of the lattice in its vicinity The mechanisms of this disorder may be illustrated with the aid of Fig 12 which represents schematically a water lattice based on H bonds The circles with extended radii represent water molecules the extended radii are H bonding directions A typical water molecule labelled A will execute torsional oscillations involving the bending of the H bonds between it and its neighbors as indicated by the arrowed lines This is describable in terms of a potential energy curve for the oscillation Consider now a second water molecule B adjacent to a nonpolar solute C The energy of interaction of B and C due to van der Waals forces will be

negative entropies of solution of nonpolar solutes in water as compared to other liquids, and the very large positive partial molar heat capacities of these solutes in aqueous solution Frank and Evans (1945) concluded that the solutes produce a stabilization of low entropy structured domains icebergs of water molecules in their vicinity The stabilization is greater the less polar the molecule The significance of this stabilization for dynamical processes has been explored by Frank (1958) who attributed the increase in dielectric relaxation time in nonelectrolyte solutions to the stabilizing influence of these molecules The pattern of diffusion in liquid water appears to be explicable on a common basis with these other anomalous properties if it is assumed that the stabilized water structures associated with nonpolar groups are able to depress the mobility of the solute That this depression in mobility is greater than that produced by solute solvent H bonding is indicated by the inversion of the pattern of specificity found in the other H bonding liquids

Chemical Specificity in Cytoplasmic Diffusion

The mechanistic basis for the argument that cellular nonelectrolyte transport is a bulk diffusional process lies in the hypothesis that the water of cytoplasm forms an extensive ordered lattice stabilized by its interaction with protein The stabilization of orientation of H bond directions in this lattice reduces the possibility of the facilitated activation in diffusion which accounts for the low chemical specificity observed in liquids For this reason a formulation of diffusion in cytoplasm in terms emphasizing its resemblance to that in crystalline solids seems justified

In the simplest rigid crystalline model of diffusion one can recognize two contributions to the unit process hole formation in the lattice and the freeing of a molecule from its lattice position to occupy the hole (Jost 1952 Ch 3) This simple picture is most closely represented by self diffusion In it the activation involves the energy of hole formation E_0 which for a molecular crystal may be taken as roughly equal to the energy of sublimation and the energy E_1 required for the diffusing molecule to surmount the energy barrier between equilibrium positions The energy of vaporization of the water in cytoplasm must lie in the same range as that of water and ice 10 to 11 kcal/mole The additional energy required to move a molecule into the hole is more difficult to assess but one may assume that the H bond breakage

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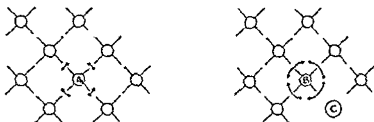


Fig. 12. A schematic water lattice based on H bonds. A, a water molecule in an undisturbed volume of the lattice. B, a water molecule adjacent to a nonpolar solute. C, For additional description see text.

small in comparison to the energy of a H bond, it will also be relatively insensitive to the orientation of molecule B. The potential energy of oscillation of B will therefore be smaller than that of A, so that B will execute oscillations of greater amplitude or may rotate completely. The oscillations of molecules neighboring B are cooperatively linked to that of B: as B turns, its H bonding directions will deviate greatly from linearity with those of its neighbors, so that the H bonds will weaken and the potential energy of oscillation for the neighboring molecules will be smaller. They then will execute wider oscillations or will rotate. Thus, a region of disorder or lattice collapse in the neighborhood of a nonpolar solute could be established.

A second mechanism will reinforce this: the nearest neighbors of molecule C are no longer tetrahedrally H bonded. It would be energetically preferable if a new equilibrium configuration of these molecules, involving an increase in H bonding, and consistent with the continued existence of the other H bonds in the system, could be realized. Such configurations are possible in liquid water because the orientational freedom permits a wide variety of configurations to coexist. However, in the more ordered system of cytoplasm such new configurations would be unlikely to match the structure imposed by the protein and thus would necessitate the substantial breakage of other H bonds. The opposing influences will result in increased disordering in a manner much like that operative in the disordered region beyond the primary hydration shell in ionic solutions in liquid water (Frank and Evans, 1945; Wang, 1954).

The region of lattice collapse would be, from preceding arguments, one in which diffusion is faster and the activation energy smaller

Among the classes of compounds involved in the present study the relatively nonpolar alcohols may be expected to show the greatest disordering effect This is consistent with the observed diffusional param ters Some notion of the magnitude of the effect is indicated by the fact that the activation energy for n butanol in cytoplasm 6 4 kcal/mole is only slightly greater than the 5 3 kcal/mole (calcula ted from data tabulated by Johnson and Babb 1956) for its diffusion in water

The purpose of this section has been to provide a framework in which the very great specificity of cellular transport can be under stood The range of this specificity is embodied in the diffusional parameters of thiourea and *n* butanol which differ by a factor of 50 fold in D_E and 9 kcal/mole in activation energy In terms of the model offered these differences are explicable in that the polar thiourea must diffuse by the breakage of strong H bonds in a medium of substantial lattice integrity characterized by a high barrier to kinetic processes while *n* butanol as a relatively nonpolar solute diffuses under cir cumstances that require less bond breakage in a local environment which has been disordered in the direction of liquid water

Dimensional Specificity

The fact that the addition of alkyl groups to a molecule increa es its rate of cytoplasmic diffusion seems explicable in terms of the preceding discussion of chemical specificity In the first place the addition of each nonpolar group decreases the number of potentially supportive H bonds relative to the volume the molecule must occupy in the lattice Secondly the steric limitations imposed by the presence on a molecule of bulky nonpolar groups tends to prevent the formation of linear H bonds between the molecule and the lattice For these reasons the same mechanisms that distinguish molecules of equal size as reflected in chemical specificity may be expected to be influenced by varying alkyl groups in an homologous series of compounds However there is insufficient data of diffusional parameters to permit further examination of the extent of this influence

While the phenomenon of a larger particle diffusing more rapidly than a smaller is rather uncommon in liquids it is a frequent feature of diffusion in latticed systems In such systems the fit of a solute into the lattice has a profound effect on the diffusion coefficient

Those solutes which most closely resemble the normal components of the crystal or which can form new compatible crystal structures provide the minimal distortion and diffuse more slowly than those for which incompatibilities exist When increasing size is a measure of incompatibility concomitant increasing diffusion coefficients can be observed Numerous examples of this are to be found in standard references on diffusion

A Comment on Narcosis

A relationship between nonelectrolyte transport and cellular narcosis has long been observed, in that both the rate of transport and narcotic potency are correlated with A_w^n It is therefore not unnatural that an hypothesis such as the present one which deals with one of these phenomena, should have implications for the other Within the framework of the present hypothesis such common bases as the phenomena may have must arise from the interaction of nonpolar materials with the stabilized water lattice of cytoplasm A common mechanism can be adduced if as suggested by several authors (see p 36) the presence of a well ordered water lattice plays an important role in a number of physiological processes One would then expect regions of disorder in the lattice to impair the operation of these processes a mechanism of cellular narcosis is thus provided by the phenomenon of disordering of the water lattice by nonpolar solutes In this formulation the correlation observed between narcosis and transport is explicable in the dependence of both on lattice structure disordering

While examination of the ramifications of this view must be deferred to another publication two recent papers of immediate relevance because they deal with the interaction of nonpolar solutes with cellular water must be noted Pauling (1961) has suggested that microcrystals of gas hydrates involving the anesthetic gas and stabilized by cellular components form in cytoplasm Miller (1961) has offered a model in which anesthetic gases enter cavities in icebergs which pre exist in the vicinity of proteins or surfaces in the cell and new icebergs form to maintain an iceberg-liquid equilibrium the overall effect is an increase in the ice cover of proteins and surfaces We have argued that the forces underlying nonelectrolyte interactions with water will in the protein stabilized water lattice of cytoplasm give rise not to

crystalline regions but to regions of disorder Two points lie at the crux of this difference of view These are whether substantial amounts of unstabilized water exist in cytoplasm and whether the dominant stabilizing influences in cytoplasm are compatible with the requirements of water structures associated with a wide variety of anesthetic substances of different sizes and functional groupings Studies on the solubility of nonelectrolytes in cells now in progress should help resolve this problem

In respect to transport the Pauling and Miller models would seem to suggest in parallel with the diffusional properties of nonelectrolytes in liquid water that the diffusion of nonpolar materials in cytoplasm will be selectively slowed this of course is at variance with the evidence of the present work

Appendix I

As indicated in Chapter 2 (p 23) it can be shown using the model of a *single cylindrical fiber with resistive surface* that the relationships of intercept slope and rate constant preclude the possibility that the rate of flux is controlled by the surface barrier while the form of the flux is determined by bulk diffusion This is so because (*a*) the bulk diffusion coefficients required to provide intercepts as low as those in the experimental flux curves are very small on the order of those calculated assuming only diffusion and (*b*) the contribution of the surface process to the overall flux must be very small if the diffusional process is substantially to influence the intercept

Crank (1956) gives the total flux in a cylinder of radius a having diffusion coefficient D and a resistive surface with permeability coefficient P Taking without loss of generality the external concentration as zero and letting $h = P/D$ we find for the mean fractional concentration

$$\frac{\bar{C}(t)}{C_0} = 4(ah) \sum_{j=1}^{\infty} \frac{e^{-D\beta_j t/a}}{\beta_j[\beta_j + (ah)^2]}$$

where β_j are the roots of

$$\beta J_1(\beta) = ah\, J_0(\beta)$$

Plotted semilogarithmically this equation shows a limiting slope s_e given by

$$s_e = D\beta_1/a$$

The intercept I of the linear portion at $t = 0$ is given by

$$I = \frac{4(ah)^2}{\beta_1[\beta_1 + (ah)^2]}$$

Taking 10^{-3} sec^{-1} as a typical value for the experimentally determined slope and 3×10^{-3} cm for the cellular radius we get

$$D\beta_1 = 9 \times 10^{-9} \text{ cm /sec} \qquad \text{(A1)}$$

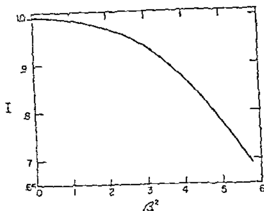


Fig 13 Intercept I as a function of β_1^2 in the combined permeation-diffusion model

as a condition which D and β_1 must satisfy The second condition is that the intercept I be substantially less than 1 Depending on the value of ah β_1 ranges from 0 (infinite D) to 5 783 (infinite P) I varies simultaneously from 1 to 0 692 In Fig 13 I is plotted as a function of β_1^2 I remains very nearly equal to 1 for small values of β_1 and does not begin to change much until β_1 reaches the range of 3–4 Values of D in the range 10^{-5} to 10^{-8} cm^2/sec which from Eq A1 give a range of β_1 of 9×10^{-4} to 0 9 will have almost no effect on the intercept and so cannot lead to curves of the form observed experimentally It is not until D becomes smaller than 3×10^{-9} cm^2/sec that a large effect on the intercept appears Similar results can be obtained using other values of the slope as well as the true mean cellular radii Thus the combined permeation diffusion model requires bulk diffusion coefficients of the same magnitude as those calculated from the simple bulk diffusion model

Examination of the permeation diffusion process from another point of view shows that the contribution of the surface process to the overall flux must be small if the intercept of the flux curves is substantially lower than unity We take the ratio of the calculated slope s to the value s^P for the pure permeation model (i e $D = \infty$) as an index of the effect of diffusion in the combined model similarly we take the ratio of s to the value for the pure diffusion model s^D ($P = \infty$) as an index of the influence of permeation Then we have

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$$\frac{\bar{C}(t)}{C_0} = 4(ah) \sum_{j=1}^{\infty} \frac{e^{-D\beta_j t/a}}{\beta_j[\beta_j + (ah)\ ]}$$

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$$I = \frac{4(ah)^2}{\beta_1[\beta_1 + (ah)^2]}$$

Taking 10^{-3} sec^{-1} as a typical value for the experimentally determined slope and 3×10^{-3} cm for the cellular radius we get

$$D\beta_1 = 9 \times 10^{-9} \text{ cm /sec} \qquad \text{(A1)}$$

relatively greater than its effect on the slope while the reverse is true of the effect of diffusion In other words a resistive surface capable of influencing the flux rate should have a significant effect on the intercept The experimentally determined intercepts however do not indicate the presence of such an effect rather their magnitudes are consistent with a pure diffusion model Thus the overall flux rate must be determined essentially by the bulk diffusion

$$\frac{s_e}{s_e^P} = \frac{D\beta_1^2/a^2}{2P/a} = \frac{\beta_1^2}{2ah}$$

$$\frac{s_e}{s_e^D} = \frac{D\beta_1^2/a^2}{5\ 783\ D/a} = \frac{\beta_1}{5\ 783}$$

We take further

$$\frac{I - 0\ 692}{1 - 0\ 692} \quad \text{and} \quad \frac{1 - I}{1 - 0\ 692}$$

as indices of the approach of the intercept I to its value for pure diffusion and pure permeation respectively In Fig 14 s_e/s_e^P and $(I - 692)/308$ are plotted in Fig 15 s_e/s_e^D and $(1-I)/308$ From these figures we may see that the effect of a resistive surface on the intercept is

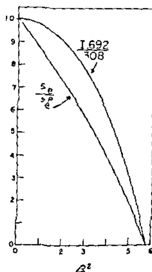


Fig 14

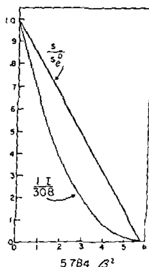


Fig 15

Fig 14 *The effect of diffusion in the combined permeation–diffusion model and the approach of the intercept I to its value for pure diffusion as functions of β_1^2*

Ordinate s_e/s_e^P and $\frac{I - 0\ 692}{0\ 308}$

Fig 15 The effect of permeation in the combined permeation–diffusion model and the approach of the intercept I to its value for pure permeation as functions of β_1^2

Ordinate s_e/s_e^D and $\frac{1 - I}{0\ 308}$

ever seen to occur This observation is consistent with others in showing that insult to the protoplasm brought about profound local changes In fact it was only rarely that a micropipette could be introduced without causing sufficient disturbance of the protoplasm to totally prevent the movement of the oil drop The experiments which Pieser considered as significant for the measurement of viscosity appear to be those injections in which the front along which the damage from micropuncture was propagated was behind the oil drop at the instant it was freed from the micropipette The movement of such a front is almost certainly associated with a local contracture of the contractile elements as they denature Such a process will result in an asymmetrical force on the oil droplet and will cause it to be pushed along as the front progresses That the oil droplet itself may as it is squeezed through the protoplasm bring about a trail of denaturation so that the process is self perpetuating can only be postulated What Pieser s experiment appears to measure is the rate of propagation of a change of muscle protoplasm from one metastable state to another

Such difficulties notwithstanding numerous observations of the behavior of protoplasm have indicated that though in special conditions it may be quite fluid its usual viscosity is high Seifriz (1936 Ch 13) places the usual value as 800 to 5000 centipoise and considers extremely high values admissible following gelation Allen s (1961) estimates for the endoplasm of the amoeba based on a variety of subtle and carefully considered techniques is in agreement with this order of magnitude

Appendix II

There are many difficulties associated with the measurement of protoplasmic viscosity. These have been reviewed by R. D. Allen (1961). It is clear from this review that protoplasmic viscosity is highly non-Newtonian and that transient rigidity under mechanical stress may also be observed. When one considers in addition the structural and functional complexity of protoplasm, the difficulty of interpreting measured values of viscosity becomes understandable.

An attempt at the measurement of cellular viscosity (Rieser 1950; see also Heilbrunn 1956, Ch. 2) of special interest to the present work because it was performed in muscle furnishes an example of the inherent difficulties. In this experiment a drop of oil about 20 μ in diameter was injected into a frog muscle fiber. Occasionally the drop would rise through the protoplasm and its rate of rise could be observed. Assuming that the rise was solely under the influence of gravity, the viscosity of the medium could be calculated. A mean viscosity of about 29 centipoise was obtained (less than that of light machine oil), a value diagnostic of a fluid protoplasm. That this conclusion is not acceptable may be seen from the following.

Striated muscle is about 18 per cent protein by weight and of this at least 57 per cent is actomyosin or its components and tropomyosin, whose viscosity increments as well as ability to form gels at low concentration argue against low values for cytoplasmic viscosity (Edsall 1943, Ch. 21; Ling, 1962, Ch. 3; Szent-Gyorgyi 1951, Ch. 8). Moreover, the contractile elements in muscle are known to be in a latticed structure held in a three-dimensional array of linkages, an arrangement suggestive of substantial solidity. Additional contradictory evidence can be derived from Rieser's experiment itself, for he found that the injected oil drop was not round as expected in a fluid but ovoid. This shows the system to be sufficiently solid to permit the maintenance of an asymmetrical force.

How can one account for the rapid movement of the oil drop? The resolution of the problem seems to lie in the observation that the movement of the oil drop was irreversible. If the fiber was turned 180° following the passage of the drop, no reversal of direction was

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VASCULAR REACTIVITY IN CATS DURING INDUCED CHANGES IN THE ACID-BASE BALANCE OF THE BLOOD

BY

STELLAN BYGDEMAN

STOCKHOLM 1963

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CONTENTS



CHAPTER I

INTRODUCTION

The cardiovascular system is known to be affected in many ways by an alteration in the acid base balance of the blood The change in the acid base balance may affect the heart directly, it may act peripherally on the blood vessels it may act on the central nervous system or it may have some reflexogenic action Many studies have dealt with various aspects of this problem and a complete review of the literature is beyond the scope of this work This introductory chapter will therefore deal primarily with one aspect of the problem namely the influence of a shift in the acid base balance of the blood on the cardiovascular effects of vasoconstrictor stimuli

PAGE and MCCUBBIN (1954) have pointed out that the extent to which vessels constrict or dilate and the extent to which the myocardium is stimulated or depressed depends not only on nervous stimuli or on the presence and concentration of humoral substances but also on the responsiveness or *reactivity* of the different components in the circulatory system When the responsiveness of the whole circulatory system was being considered by these authors the term *cardiovascular reactivity* was used The same terminology has been adopted in this study

Four main types of acid base shift in the body are usually considered respiratory acidosis and alkalosis primarily due to changes in the CO_2-tension of the blood and non respiratory or metabolic acidosis and alkalosis primarily due to non respiratory changes in the bicarbonate concentration of the blood These alterations in the acid base balance may be more or less compensated by secondarily induced changes in respiratory activity in kidney function or in both

Systemic blood pressure responses to vasoconstrictor substances during acid base shifts in the blood

Previous workers have studied mainly the influence of various vasoconstrictor agents on the arterial blood pressure during experimentally induced alterations in the acid base balance

Respiratory acidosis was usually induced by increasing the percentage of CO_2 in the inspired gas mixtures In some instances controlled artificial ventilation was also employed Other investigators have induced acidosis by de

naline after injections of NaH_2PO_4 i v and an increased effect after Na_2CO_3 KEITH SNYDER and CAMPBELL (1960) and SNYDER KEITH and CAMPBELL (1960) have also reported decreased blood pressure responses to adrenaline during non respiratory acidosis NAHAS et al (1960) as well as MEFSMANN et al (1961) observed further that the pressor effect of noradrenaline which had been decreased by respiratory acidosis could be improved if the arterial blood pH was restored to the normal value through i v infusions of either tris hydroxymethylaminomethane (THAM) or $NaHCO_3$ However RADER et al (1961) reported that the systemic pressor effects of adrenaline were unaltered by non respiratory alkalosis

Reactivity of some vascular beds in the peripheral circulation during acid base shifts

Observations of blood pressure responses to vasoconstrictor agents give information only about the overall reactivity of the circulatory system They allow no conclusions to be drawn concerning the reactivity of either local vascular beds or of the heart For this reason many workers studying individual vascular beds have altered the acid base balance of the perfusion fluid and studied the effect of this alteration on the reactivity to vasoconstrictor substances A decreased effect of adrenaline has been observed when *non respiratory* acidosis is present in the hind leg of the frog (ALDAY REDONNET 1920 SCHMIDT 1921 HULSE 1922, MENDICI 1924 OKUYAMA 1925) and in the rabbit ear (ALPERN 1924 LEITES 1925) On the other hand SANO in 1933 found an increased effect of adrenaline in the perfused rabbit ear when the pH of the perfusate was decreased

The majority of the preceding experiments were performed on poikilothermic animals Salt solutions were employed as perfusates and unphysiological changes in pH were induced More recent investigators employing moderate acid base shifts have encountered difficulties in confirming those earlier results BOHR and MCVAUGH (1959) and BOHR (1962) perfused the rabbit ear and rat hind leg preparations with Krebs s solution at a constant rate of flow and evaluated the influence of changes in pH on the effect of adrenaline In their experiments a decrease in pH from 7 4 to 6 9 *respiratory* or *non respiratory* increased the response to intraarterial (i a) adrenaline injections while an increase in pH from 7 4 to 7 9 decreased the response In the dogs foreleg perfused *in situ* with the animals own blood the effect of i a injections of adrenaline remained unchanged during 7 5 minutes ventilation with 20% CO (FLEISHMAN SCOTT and HADDY 1957) However while studying the vascular bed of the carotid artery in dogs NASH

creasing the ventilation The accompanying hypoxia in these cases makes the analysis of the results rather difficult, however

Ventilation of the dog with 10—30 % CO (PAGE and OLMSTED 1951, HOULE et al 1957) or of the human with 10% CO (CAMPBELL et al 1958) diminished the blood pressure response to administered adrenaline A reduced response of the blood pressure to noradrenaline and angiotensin during ventilation with 10—30% CO has been reported in dogs (PAGE and OLMSTED 1951, HOULE et al 1957, MEESMANN, BRAASCH and HERBERG 1961) The pressor effect of adrenaline and noradrenaline is also diminished *during hypoventilation in normal cats* (DUZAR and FRITZ 1924, DUNER and LUIER 1959), in spinal cats (STAVRAKY 1942) and in normal dogs (apneic oxygenation) (NAHAS, LIGOU and MEHLMAN 1960) PAGE and OLMSTED (1951) found that of the vasopressor substances tested the response to adrenaline was most easily abolished, noradrenaline was next and angiotensin was the least easily abolished However, MEESMANN et al (1961) reported that the ratio between the vasopressor doses of angiotensin and noradrenaline changed from 1 5 to 1 1 25 during ventilation with 15% CO

PAGE and OLMSTED (1951) suggested that the refractory state observed during respiratory acidosis was a result of inhibitory impulses emanating from stimulation of sympathetic ganglia BURGET and CRISLER (1927) observed in cats that the blood pressure responses to stimulation of the sympathetic chain in the neck and to i v adrenaline injections varied in the same way and at the same time as changes in the pH of the blood They suggested that these variations in blood pressure responses were due to alterations in the irritability of the myoneural junction

From the above survey it seems that respiratory acidosis usually diminishes the effect of vasoconstrictor substances However, the reports of the influence of *respiratory alkalosis* are more conflicting An increased pressor effect of adrenaline has been observed during hyperventilation in normal cats (DUZAR and FRITZ 1924), in pithed cats (BURGET and VISSCHER 1927), in dogs (RADIE, KEITH and CAMPBELL 1961) and in humans (CAMPBELL et al 1958) On the other hand a diminished response to adrenaline during hyperventilation has been reported by STAVRAKY (1942) in pithed cats and by BALINT and GOLDSCHMIDT (1922) and BREHME and POPOVICIU (1926) in humans

Earlier workers studying the influence of *non respiratory* shifts in the acid base balance on cardio-vascular reactivity have also produced conflicting reports PAGE and OLMSTED (1951) did not observe any change in the pressor effects of adrenaline, noradrenaline or angiotensin during infusions of 0 1 N HCl COLLIP (1921) however, noted a diminished response to adre

naline after injections of NaH_2PO_4 i.v. and an increased effect after Na_2CO_3. KEITH, SNYDER and CAMPBELL (1960) and SNYDER, KEITH and CAMPBELL (1960) have also reported decreased blood pressure responses to adrenaline during non respiratory acidosis. NAHAS et al. (1960) as well as MEESMANN et al. (1961) observed further that the pressor effect of noradrenaline which had been decreased by respiratory acidosis could be improved if the arterial blood pH was restored to the normal value through i.v. infusions of either tris hydroxymethylaminomethane (THAM) or $NaHCO_3$. However RADER et al. (1961) reported that the systemic pressor effects of adrenaline were unaltered by non respiratory alkalosis.

Reactivity of some vascular beds in the peripheral circulation during acid base shifts

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The majority of the preceding experiments were performed on poikilothermic animals. Salt solutions were employed as perfusates and unphysiological changes in pH were induced. More recent investigators employing moderate acid base shifts have encountered difficulties in confirming those earlier results. BOHR and MCVAUGH (1959) and BOHR (1962) perfused the rabbit ear and rat hind leg preparations with Krebs's solution at a constant rate of flow and evaluated the influence of changes in pH on the effect of adrenaline. In their experiments a decrease in pH from 7.4 to 6.9 (*respiratory* or *non respiratory*) increased the response to intraarterial (i.a.) adrenaline injections while an *increase* in pH from 7.4 to 7.9 decreased the response. In the dog's foreleg perfused *in situ* with the animal's own blood the effect of i.a. injections of adrenaline remained unchanged during 7.5 minutes ventilation with 20% CO₂ (FLEISHMAN, SCOTT and HADDY 1957). However while studying the vascular bed of the carotid artery in dogs NASH

and HEATH (1961) observed that *respiratory acidosis,* induced by ventilating with 30% CO_2, diminished the vasoconstriction produced by i a injections of noradrenaline In agreement with these results BYGDEMAN and EULER (1962), working with cats, reported that ventilation with 6 5—20% CO_2 diminished the noradrenaline induced vasoconstriction in the vascular bed supplied by the femoral artery Hyperventilation increased the peripheral effect of noradrenaline (NASH and HEATH 1961)

In vitro experiments employing isolated aorta strips have made it possible to study the effect of noradrenaline on vascular smooth muscle during different acid base shifts The noradrenaline induced contractions of strips from rat aorta were found to decrease by 43% during "*respiratory acidosis* (pH 7 19) (TOBIAN, MARTIN and EILERS 1959) The result also suggested that the diminished effect was due mainly to the change in pH and not to the concomitant increase in CO_2-tension Using strips from rabbit aorta and a ten times greater noradrenaline concentration (10 μg/l) WILLIAMSSON and MOORE (1960) failed to observe an altered response when the pH was changed from 7 14 to 7 56 The response to noradrenaline was diminished equally if there was a greater fluctuation in pH in either direction (pH 6 8 or 7 7)

The function of the heart is impaired during acidosis This is most obvious in the isolated preparations (JERUSALEM and STARLING 1910, MARKWALDER and STARLING 1913, NAHAS and CAVERT 1957, MCELROY et al 1958) but in the intact heart a slight reduction in stroke work has also been observed (MONROE FRENCH and WHITTENBERGER 1960) A state of acidosis will also decrease the effect of noradrenaline on ventricular isometric tension (DARBY et al 1960)

From the investigations mentioned in the preceding review it is evident, that alterations in the acid base balance of the blood can markedly influence the reactivity of various components of the circulatory system to vasoconstrictor substances In spite of the conflicting reports it is generally assumed that the systemic blood pressure responses to i v injections of vasoconstrictor substances is enhanced by alkalosis and hindered by acidosis However, the nature of the altered reactivity is still uncertain This may account for the earlier practice of measuring systemic blood pressure responses and using them as the only index of cardiovascular reactivity As previously noted an alteration in the acid base balance will by itself induce major cardiovascular adjustments A failure to consider these when observing a changed blood pressure response to a vasoconstrictor substance may well invalidate any conclusion relating to cardiovascular reactivity To illustrate this point it can be

mentioned that, for example during respiratory acidosis some vascular beds constrict while others dilate leading to an altered distribution of the i v in jected substance (for references see chapter VII) By means of such a shunt a larger portion of an injected compound may be directed to a vascular bed with a basically low sensitivity resulting in a derreased blood pressure re sponse even though no change in the reactivity of any area has occurred

In vitro experiments on isolated vascular smooth muscle are not subject to these errors Employing *in vitro* techniques the influence of well controlled changes in the acid base balance on the noradrenaline induced contractions can be studied However it must always be remembered that erroneous con clusions can be drawn if the results obtained *in vitro* on smooth muscle from major vessels are regarded as the same as one would expect from the resist ance vessels *in vivo* Also the results of the present study suggest t iat the re activity of different peripheral vascular beds can change in quantitatively different ways during acid base shifts This observation will of course in crease the possible errors when generalizing about results obtained on smooth muscle strips from major vessels

In spite of the efforts to elucidate the *in vivo* mechanisms governing the altered respon es to vasoconstrictor substances during shifts in the acid base balance of the blood many basic questions remain Since this problem is not only of physiological interest but also has most important clinical implica tions the present study was undertaken

In the present investigation the altered reactivity of the circulatory system during respiratory acidosis has been studied Special attention has been given to the reactions of some peripheral vascular beds The effect on vascular re activity of some types of acid base shift other than respiratory acidosis have also been included in the study

The results will be presented in three sections In the first part (chapter IV) the effect of noradrenaline on the systemic blood pressure and the re sponse of some peripheral va cular beds to i a injections of noradrenaline were observed during respiratory acidosis The importance of an intact sym pathetic nervous system for the observed changes in reactivity was evaluated The second part (chapter V) contains the results obtained with a vasocon strictor other than noradrenaline namely angiotensin and with sympathet ic nerve stimulation In the third part (chapter VI) the results obtained dur ing respiratory alkalosis and non respiratory changes in the acid base balance have been summarized

Preliminary reports of some of the results included in the present study have been published (BYGDEMAN and EULER 1962 BYGDEMAN 1963 a b and c)

CHAPTER II

METHODS

Experiments were undertaken to determine the influence of individual or combined changes in pH and CO_2 tension on the reactivity of various peripheral vascular beds to different vasoconstrictor stimuli. A satisfactory analysis of the local haemodynamic events in a vascular bed necessitates determination of at least two important basic parameters: a) the volume of blood circulating through the vascular region and b) the arterio-venous blood pressure gradient across the vascular bed. In practice the venous pressure is taken as zero and, accordingly, the systemic arterial blood pressure or the perfusion pressure is generally used for the calculation of resistance to blood flow offered by the vascular region. According to GREEN et al. (1944) the resistance to flow in a vascular bed can be expressed as the ratio between arterial pressure and blood flow through the bed.

$$1 \text{ unit of peripheral resistance } 1 \text{ PRU} = \frac{1 \text{ mm Hg}}{1 \text{ ml/min}}$$

When the effects of different vasoconstrictor stimuli on a peripheral vascular bed are compared quantitatively it is usually the induced changes in resistance that are calculated and compared. There are three different ways to obtain the data necessary for such calculations:

a) by perfusing a vascular bed at a constant pressure and measuring changes in flow.

b) by using a constant flow and measuring changes in perfusion pressure.

c) by measuring both flow and pressure, neither of them being held constant at the time of the vasoconstrictor stimuli.

The first two ways have been widely used. The use of the third method can give in many cases erroneous results even in a qualitative respect (GREEN et al. 1944). The first two methods were compared by BURTON and STINSON (1960). Their results suggest that the constant flow perfusion technique produces a better relationship between the tension developed by the smooth muscle of the vascular wall in response to a pressor stimuli, the so called "primary response," and the change in resistance. GREEN et al. (1944) plotted the ratio between the peripheral resistance of a vascular bed in a constricted state and the resistance in a control state at the same perfusion pressure against the perfusion pressure. The resulting curve showed more

fluctuations than if the resistances at the same flow rate had been compared These results therefore suggested that the constant flow perfusion technique was preferable in experiments quantitatively comparing the peripheral effects of vasoconstrictors where different degrees of vascular resistance often prevailed This method adopted throughout this study may also possess some marked disadvantages (FOLKOW 1953) These will be discussed later In the present work the perfusion pressure responses to different vasoconstrictor stimuli during the various experimental conditions were used directly for all comparisons The peripheral resistance was seldom calculated An estimation of the *reactivity* of a perfused vascular bed during different conditions was therefore based on the response of the perfusion pressure when the flow was kept constant

It is well known that the acid base balance of the blood can be expressed in terms of three variables the hydrogen ion concentration $[H^+]$ the concentration of carbonic acid $[H_2CO_3]$ and the bicarbonate concentration $[BHCO_3]$ The relation between these variables may be represented by the following equation

$$[H^+] = K\frac{[H_2CO_3]}{[BHCO_3]}$$ in which K is a constant

The concentration of H_2CO_3 is directly proportional to the CO_2 tension Since this is practically the same in the alveolar air as it is in the arterial blood (KROGH and KROGH 1910) at least during constant ventilation (MATELL 1963) changes in the acid base status can be adequately followed by continuously recording the end tidal CO_2-tension together with the arterial blood pH However in the majority of experiments dealing with the effects of respiratory acidosis the CO_2-tension has not been continuously recorded Observed changes in vascular reactivity were therefore correlated with the arterial blood pH and with the $\% CO_2$ in the inhaled gas mixture

1 *Operative procedure*

Successful experiments were performed on 90 cats They weighed between 1 6 kg and 5 0 kg The animals were anaesthetized with sodium pentobarbital (Nembutal® Abbott) 35 mg/kg b w injected intraperitoneally During the experiments small additional doses of sodium pentobarbital or urethane (20% solution) were given as needed in order to maintain a uniform depth of anaesthesia No difference was observed between the experiments in which urethane was used and those employing sodium pentobarbital

The animals were placed on a thermostatically controlled operating table

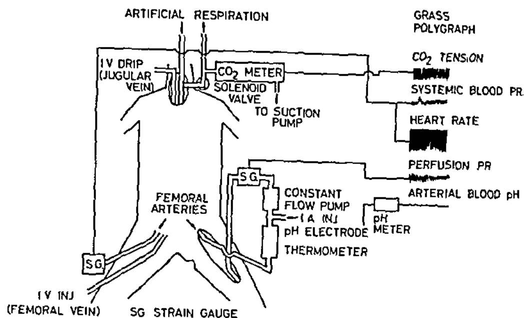


Fig 1 General scheme of the experimental set up used in most experiments

The body (blood) temperature was kept at 38°C ± 0 5°C The general principle of the preparation of the animals is illustrated in Fig 1

The trachea was cannulated and spontaneous breathing was permitted during the operative procedure

Injections were given intravenously through a cannula inserted in the right femoral vein In experiments where two venous cannulae were needed the second one was placed in the left jugular vein

Vascular reactivity was studied employing the constant flow perfusion technique *The following peripheral beds were perfused in vivo using* the cats own blood

a) skinned hind leg
b) unskinned hind leg
c) kidney

In perfusion experiments on the skinned hind leg or the unskinned hind leg, the left leg was prepared at the proximal part of the thigh Special care was taken to exclude all possible routes of collateral circulation The method has been employed previously in this laboratory (Yamada and Åstrom 1959) The muscles were divided by blunt dissection into four portions and these together with areas of the skin, were tied off with individual rope ligatures A fifth ligature was interwoven throughout the other four and tied around the bone and adjacent muscles The femoral and sciatic nerves as well as the

femoral vessels remained intact The bone marrow cavity of the femur was plugged with absorbent paper through a hole drilled at the same level In the first series of experiments the leg was subsequently skinned and the paw tied off just above the ankle joint leaving the femoral artery to perfuse mainly skeletal muscles The leg was kept moist during the skinning procedure with warm Ringer solution and during the experimental period drying was prevented by sewing the skin back in place again Both in the skinned leg preparation and in the intact leg preparation the femoral artery was cannulated proximally and distally The blood flow was exteriorized in a polyethylene loop containing a thermometer a pH electrode (Radiometer GK 264) and a pump (Sigmamotor) To prevent cooling of the blood during the passage through the loop the section containing the pH electrode and the thermometer was surrounded by a constant temperature water bath (38°C) The remaining part of the loop was passed through a sack through which air warmed to approximately 40°C was blown

In the kidney perfusion experiments the left renal artery was cannulated with a polyethylene catheter close to the aorta and the kidney perfused with blood from the ipsilateral femoral artery via a polyethylene loop arranged in the same way as above

In experiments where carotid occlusion tests were performed both common carotid arteries were dissected free at the neck and the vagus nerves were cut

In one series of experiments the left sympathetic chain was dissected free in the lumbar part Sympathetic denervation of the ipsilateral lower extremity was carried out by dissecting and removing a section of the lumbar sympathetic chain including 2—3 ganglia at the level of the fourth to sixth lumbar vertebrae Electrical stimulation of the chain was performed by cutting the chain at the level of the fourth lumbar vertebra and placing a bipolar platinum electrode on the peripheral end In this series of experiments the adrenal glands were denervated by bilateral section of the splanchnic nerves Difficulty in denervating the right adrenal was occasionally encountered and in such cases the venous outflow from the gland was ligated instead

2 *Recording and measuring techniques*

For continuous recording of the measured physiological functions a 4 channel polygraph (Grass) was used

Systemic arterial blood pressure was measured with a Statham strain gauge pressure transducer connected to a polyethylene tube filled with saline and inserted in the right femoral artery

Perfusion pressure was measured with another strain gauge transducer connected to the exteriorized arterial loop distal to the pump

Heart rate was determined with an interval recorder (GOLDSCHMIDT and LINDGREN 1962) via impulses from the systemic blood pressure channel of the Grass instrument, when end tidal CO tension was not recorded

Arterial blood pH was measured with a pH electrode inserted in the exteriorized arterial loop as described above and connected to a pH meter (Radiometer 1 HM 22) The pH electrode was shielded by adding sodium chloride to the water bath and grounding it to earth To prevent the blood from entering the calomel part of the electrode during the experiment and thus interfering with the readings, its upper end was placed under a pressure slightly above the blood pressure This pressure was supplied through a plastic tube connected to the loop about 5 cm before the electrode and filled with saline in one part and saturated potassium chloride in the other, the liquids being separated by a thin latex finger stall The pressure difference over the electrode was thus determined by the blood pressure drop in the 5 cm part of the loop The electrode was calibrated with 0 067 M phosphate buffers, pH approximately 7 38 and 6 84 ± 0 02 The absolute values of these buffers were regularly checked against commercially available buffers, pH 7 381 ± 0 005 and 6 840 ± 0 005 at 38°C (Radiometer) The calibrations were carried out at 38°C employing approximately the same rate of flow as the blood Minor changes in flow did not, however, change the recorded pH values

End-tidal CO tension was determined by continuously drawing the expiratory air through the 'micro catheter sample cell' of a CO infra red analyzer (Beckman Spinco, Model LB 1) at a sped of 200 ml/min The highest value recorded during each breath was taken as the end tidal CO tension The short response time of the analyzer, 90% deflection in 0 1 sec as well as the constant artificial ventilation made this approach acceptable To diminish the space between the tracheal cannula and the sensing unit of the analyzer the expiratory valve of the Starling pump was replaced with a solenoid valve placed as near the branch of the cannula as possible To the other opening of the valve a 40 cm long tube with an inner diameter of 7 mm was connected The gas was drawn from the first part of this tube and passed through the analyzer The tube thus served as a reservoir which supplied the analyzer with expiratory air during the inspiratory phase of the respiratory cycle The opening of the valve was monitored by a microswitch and was synchronized to operate with the pump The CO meter was calibrated by using a series of known mixtures of CO in O saturated with water vapour at room temperature

The reactivity of chosen vascular beds in the peripheral circulation was tested with three types of vasoconstrictor stimuli a) noradrenaline b) angiotensin (both these substances were injected i a in the exteriorized loop just proximal to the pump) c) electrical stimulation of the left sympathetic chain (see above under operative procedure) The i a injections were given with the help of an infusion apparatus The plunger of the syringe was uniformly pushed by means of a motor driven propelling screw In each experiment a constant injection time of 20 or 30 sec was used In the stimulation experiments the electrode was connected to a square wave generator (Grass) and stimulation was performed with a supramaximal voltage 4—15 V a duration of 10 msec and a frequence range of 0 1—8 imp/sec The duration of each period of stimulation was 30 sec

Changes in vascular reactivity concomitant with induced changes in the acid base balance of the blood were determined in two ways

a) The dose of the vasoconstrictor substance used or the stimulation frequency employed was adjusted so that the perfusion pressure responses amounted to about 50 (range 40—60) mm Hg during a period of normal arterial blood pH The change in response during a sub equent acid base shift was recorded and calculated in per cent of control response

b) The dose of the vasoconstrictor substance or the stimulation frequency was changed to obtain an increase in perfusion pressure of 50 mm Hg also during periods with altered acid base balance Employing this method the change in vascular reactivity could be expressed by the ratio between the doses eliciting this standard response However it was often difficult to find a dose giving an exact 50 mm Hg response For this reason two or more doses were often used and the dose was then calculated from the dose response curve

3 *Compounds utili ed in the study*

1 *Noradrenaline* Noradrenaline hydrochloride (l form) was employed generally in this study as a vasoconstrictor substance and was adopted as a standard substance for the comparative analysis of other vasoconstrictor stimuli The noradrenaline was freshly prepared for each experiment by diluting a stock solution with isotonic saline acidified to pH 4 with HCl Noradrenaline doses given in the text refer to the hydrochloride

2 *Angiotensin* (Hypertensin ® Ciba) The powerful peripheral vasoconstrictor effects of angiotensin are mediated via receptors different from those sensitive to noradrenaline (PAGE and BUMPUS 1961) In the present study angiotensin was compared with noradrenaline in order to eval

Perfusion pressure was measured with another strain gauge transducer connected to the exteriorized arterial loop distal to the pump

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or angiotensin until a constant perfusion pressure response was obtained During this control period the animals were ventilated with oxygen

The desired change in acid base balance was subsequently induced and the vascular reactivity of the perfused bed was determined again 20 to 40 minutes later

Respiratory acidosis was obtained by ventilating with 10% or 20% CO in O instead of pure O The gas mixtures during the experiment were kept in special Douglas bags of polyvinylchloride connected to the inspiratory valve of the Starling pump with a five way stopcock

Respiratory alkalosis was induced by hyperventilation with O_2 With the experimental procedure employed the ventilation rate and tidal volume always remained unaltered between the control and experimental period It was thus found necessary to ventilate during the control period with 3—5% CO in O according to the degree of hyperventilation to maintain the CO tension and arterial blood pH within normal levels

Non respiratory changes in the acid base balance were induced by i v infusions of 0 6 M $NaHCO_3$ or 0 2 M HCl The $NaHCO_3$ solutions were always freshly prepared to avoid transition to Na CO_3 by escape of CO

In some experiments the CO-tension in end tidal air was decreased while the arterial blood pH was kept constant (compensated non respiratory acidosis) This was accomplished by a slow infusion of HCl along with a suitable degree of hyperventilation

uate the specificity of the recorded changes in vascular reactivity during acid base shifts It was diluted with normal saline before infusion

3 *Atropine* Atropine sulphate (1%) was administered after the operation was finished in experiments involving either carotid occlusion or sympathetic chain stimulation The dose employed, 0 2—0 4 mg/kg i v has been shown to block the actions of the sympathetic vasodilator system (LINDGREN 1955)

4 *Reserpine* (Serpasil®, Ciba), 5 mg/kg, was given intraperitoneally to a series of animals 24 hours before the perfusion experiment was to be made It was given in order to prevent the effect of sympathetic vasoconstrictor impulses The reserpine treatment was tested at the end of each experiment and was considered successful if electrical stimulation of the lumbar sympathetic chain, after atropine treatment, failed to cause a vasoconstriction

5 *Succinylcholine iodide* (Celocurin Iodide®, Vitrum), a neuromuscular blocking agent with a shortlasting effect, was given in the experiments where artificial ventilation was used It was administered as a slow i v drip added to 100 ml of a 3% solution of dextran (RHEOMACRODEX®, Pharmacia) in an amount of 0 1—0 2 g The rate of the drip was adjusted so that the spontaneous respiratory movements were just abolished

6 *Dextran* (Rheomacrodex®, *Pharmacia*) *diluted with 5% glucose to* an isotonic solution (3%) was used to fill the tubes of the arterial loop and for the i v drip to compensate for fluid loss during the experiments Rheomacrodex does not change the suspension stability of the blood corpuscles (ELIASSON and SAMELIUS BROBERG 1963)

4 *Course of a standard experiment*

When the operative procedures were finished the animals were heparinized The artificial ventilation and the perfusion pump were started The ventilation was adjusted to maintain a constant pH of 7 35 —7 40 The spontaneous respiratory movements were abolished through the i v drip of succinylcholine The recording of CO tension in end tidal air was started A decreased tension was usually noticed and at the prevailing normal arterial pH this was interpreted as an indication of a decrease in bicarbonate concentration (compensated non-respiratory acidosis) developed during the operative procedure Sodium bicarbonate was injected i v to restore the normal acid base balance The perfusion pump rate was adjusted to obtain a perfusion pressure equal to the systemic blood pressure The reactivity of the perfused vascular bed was determined with repeated i a injections (into the loop) of noradrenaline

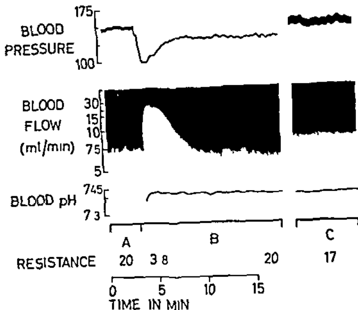


Fig 2 Cat 3 9 kg Influence of the constant flow perfusion technique on the vascular tone of the perfused vessels in the hind leg For details see text

senteric artery and the loop This technique was used in two cats and Fig 2 shows selected parts from one such experiment

During period A in this figure the arterial blood flow to the leg was directed via the normal arterial pathway At the beginning of period B the abdominal aorta was occluded and the clamp on the superior mesenteric artery released The arterial inflow had then to pass through the loop the pump being open instead of by the normal way This caused a very marked dilatation of the vessels in the limb in accordance with the observation made by FOLKOW (1953) The vasodilatation was shortlasting however and within 5 minutes the vascular resistance had returned to its previous value in spite of the fact that the blood still passed through the loop The mere passing of the blood through the loop therefore did not interfere with the basal tone of the vessels except during the first minutes This also indicated that the small contamination of the blood with potassium chloride from the pH electrode did not reach a sufficient concentration to induce vasodilatation (KJELLMER 1961) At the beginning of period C in Fig 2 the flow was adjusted to give the same perfusion pressure as at the start This reduced the vascular resistance from

CHAPTER III

INTRODUCTORY EXPERIMENTS

Perfusion device and vascular tone

It has been mentioned in the preceding chapter that the constant flow perfusion technique offers many advantages in a study of the effect of vasoconstrictor substances. FOLKOW (1953) has pointed out, however, in a critical study concerning the methods usually used in experimental work on circulatory problems that even the mere passing of the arterial blood through a pump device or flow recorder could induce an almost maximal dilatation in the cognate vascular bed. He also observed that the active reactions of the vascular smooth muscle to changes in blood pressure were depressed at the same time. He suggested that the dilatation was due to a release of ATP from injured red blood corpuscles. The passing of blood through a pump device will evidently cause some hemolysis and ATP release although the degree of the hemolysis will vary with the type of pump and connecting tubes used (CAHILL and KOLFF 1959). Because of this it seemed necessary to evaluate to what extent the basal tone of perfused vessels was influenced by the perfusing and recording devices used in the present study. This was done using principally the same method as FOLKOW. The technique therefore will be only briefly outlined. The right hind limb was chosen for the study and the skin and muscles were tightly ligated at the upper part of the thigh. The right sympathetic chain was resected at the midlumbar level and the suprarenal glands were denervated. The intestines were resected. The branches of the lower part of the abdominal aorta, of the right iliac artery and of the right femoral artery were ligated down to the level of the ligatures around the muscles (upper part of the thigh). The superior mesenteric artery and the central stump of the ligated left femoral artery were cannulated and the cannulae were connected by a polyethylen loop. In the loop the pH electrode and the thermometer were inserted. The blood flow through the loop could be regulated by the Sigmamotor pump (see chapter II). The venous outflow from the right leg was measured with a drop recorder (LINDGREN 1958) inserted in the femoral vein. The blood pressure was recorded from the caudal extension of the aorta. Through clamps on the abdominal aorta and superior mesenteric artery the arterial blood flow to the right leg could be directed either via the normal arterial pathway or via the superior me

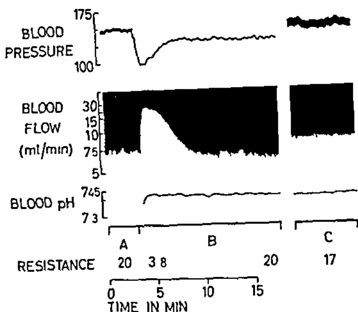


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20 to 17 units indicating a slight interference with the basal vascular tone In the second experiment the resistance was unchanged when the pump was started (increased from 10 7 to 10 9 units)

A decrease in basal vascular tone, tested with i a injections of acetyl choline, could not be observed in the perfused hind leg of cats treated with reserpine even after several hours of pump perfusion

When the choice was to be made between recording venous outflow or using a constant flow perfusion technique it was evident that both methods had advantages, which could not be combined The final reason for using the constant flow perfusion technique was that this method seemed to re flect best the changes in tension of the vascular smooth muscle The intro ductory experiments also suggested that with this set up, the damage to the blood interfered with the basal vascular tone of the perfused vessels either only slightly or not at all

Effect of noradrenaline and vascular tone

It soon became evident that during the course of the experiments the acid base shifts of the blood induced not only changes in the vasoconstrictor effect of noradrenaline but also altered the resistance of the perfused vascular bed FOLKOW and ÖBERG (1959) as well as LAVERTY (1962) have, however, shown that changes in the resistance of a vascular bed alter the effect of vasoconstrictor substances For the interpretation of the results therefore it seemed necessary to evaluate to what extent the observed changes in vascular resistance could alter the vasoconstrictor effect of noradrenaline during acid base shifts In these experiments the skinned hind leg preparation was used Changes in vascular resistance was induced by stimulation of the ipsi lateral sympathetic chain at the lumbar level The effects of i a injections of noradrenaline on the perfusion pressure were tested before and during graded sympathetic stimulation The mean dose of noradrenaline was 0 038 μg/kg (range 0 017—0 068) Fig 3 sums up the results obtained in 8 experiments on 3 cats In two cats (○—○ and ◑—◑ in Fig 3) an increased vascular tone only slightly affected the perfusion pressure responses to i a injections of noradrenaline while in the third cat (●-●) the perfusion pres sure responses decreased In this cat the control level of the perfusion pressure was 120—135 mm Hg In the other two cats the control pressure varied be tween 160 and 185 mm Hg

It was also calculated that during a period of sympathetic nerve stimula tion, which induced a 50 mm Hg increase of the perfusion pressure the test

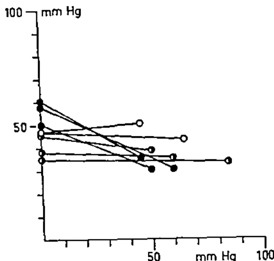


Fig 3 Effect of an increase in vascular tone induced through stimulation of the sympathetic chain on the perfusion pressure responses to i a. injections of noradrenaline in the perfused skinned hind leg The results were obtained in three cats Curves from the same cat are marked in the same way (O—O ◐—◐ or ●—●) Ordinate Increase in perfusion pressure following i a. noradrenaline injections Abscissa Increase in perfusion pressure during electrical stimulation of the sympathetic vasoconstrictor nerves

dose of noradrenaline injected i a had to be doubled (mean value) to give the same pressure response as during the control period

Determination of changes in vascular reactivity

When this study was started the question arose of how to measure changes in vascular reactivity quantitatively so that results obtained during different types of acid base shifts and in different vascular beds could directly be compared

One way was to measure the perfusion pressure response to repeated i a injections of a test dose of noradrenaline before and during an acid base shift and calculate the change This has been the method principally employed in earlier studies If we assume that during a period of acidosis the perfusion pressure response to a test dose of noradrenaline decreased by 50%, this result will be rather meaningless in a quantitative respect unless we know either the relative position of the test dose within the dose response curve or the slope of the curve For instance if we compare the results obtained

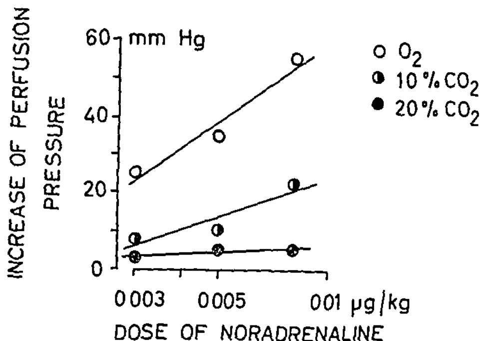


Fig 4 Cat 3 0 kg Dose response curve obtained with i a noradrenaline injections in the perfused unskinned hind leg during ventilation with O (control period) 10 and 20% CO in O

in two experiments during acidosis we cannot draw the conclusion that a 50% decrease of the perfusion pressure response in both experiments will mean the same change in vascular reactivity, unless we make the assumption that the slope of the dose response curves are the same in the two experiments

A *more meaningful measurement of a change in vascular reactivity during* an acid base shift can be obtained by determining the horizontal shift of the dose response curve

Fig 4 illustrates the relationship between different doses of noradrenaline and the perfusion pressure responses in the perfused unskinned hind leg The figure shows that during acidosis the position of the doses of noradrenaline within the dose response curve has changed The decreased slope of the curves during acidosis indicate a horizontal shift of the dose response curve to the right The magnitude of the shift cannot however be determined from the curves in Fig 4

If we assume that the only thing that happens to the dose response curve of noradrenaline during *acidosis is a horizontal shift, a part of the curve* with the same slope as that during the control period will be obtained if the doses of noradrenaline are increased so that the responses are kept basically un

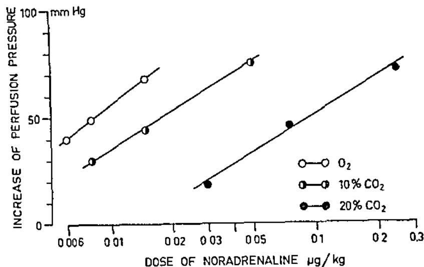


Fig 5 Cat 3 0 kg Dose response curves obtained with i a injections of noradrenaline in the perfused unskinned h nd leg during ventilation with O (control period) 10 and 20% CO in O The doses of no adrenaline were increased during vent lation with CO to keep the perfusion pressure responses at the same range as during the control period

changed This was done in the experiment from which the curves in Fig 5 have been taken Now it is pos ible to measure the horizontal shifts of the dose response curve and this measure of a changed reactivity is independent of the original slope of the curve With this method it is thus possible to make quantitative comparisons between changes in vascular reactivity obtained in different experiments The magnitude of the shift in an experiment can be expressed in different ways One way which has been used in the present study is to calculate the ratio between equieffective doses (cf GADDUM et al 1955) In Fig 5 the ratio will be the same if both doses giving for instance a 40 mm Hg or a 60 mm Hg response are compared since the curves are parallel It was not always possible however to obtain parallel curves and therefore the doses elicitating a 50 mm Hg response were used for determining the ratio

In the present study both the reduction of a response during acidosis and the ratio between equieffective doses were calculated (cf chapter II)

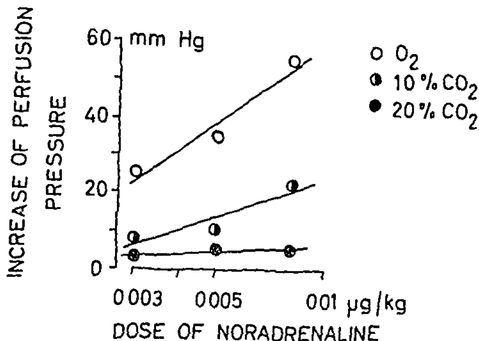


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CHAPTER IV

EFFECT OF NORADRENALINE ON THE CARDIOVASCULAR SYSTEM DURING RESPIRATORY ACIDOSIS

The systemic blood pressure responses to i v injections of noradrenaline were recorded before and during respiratory acidosis The effects of respiratory acidosis on the reactivity of peripheral vessels were studied in the skinned hind leg (muscle vessels) in the unskinned hind leg (skin and muscle vessels) and in the kidney

1 Special methods

In some experiments the distribution of the blood flow between skin and muscle vascular beds in the unskinned hind leg preparation was investigated This was made by measuring the venous outflow from a skin and a muscle vascular bed or by measuring the venous outflow only from a skin vascular bed when the arterial inflow to the leg was kept constant with the pump

When the muscle blood flow was studied the leg was skinned as described earlier and immediately above the ankle a tight ligature was applied The femoral vein was dissected free just below the inguinal ligament and cannulated

To obtain a predominate skin blood flow the saphena magna vein which drains the paw was dissected free for cannulation a few cm proximal to the ankle To prevent the blood from passing via collaterals to the saphena parva vein the latter was ligated

When the skin and muscle blood flow were recorded in the same animal both hind legs were used

The volume of blood flow was determined with the help of a closed drop chamber filled with silicon oil (Grave DC 200 viscosity 0 65 cS) connected to the venous cannula (LINDGREN 1958) For recording the drop rate a photocell operating an electronic interval recorder (GOLDSCHMIDT and LINDGREN 1962) or a Grass preamplifier model SP4 was used With the former recorder the heights of the deflections were approximately inversely proportional to the rate of blood flow After passing the chamber the blood

Correction of the acid-base balance of the blood

In the present study a mean end tidal CO_2 tension of 26 mm Hg (20—35) at a mean arterial blood pH of 7.37 (7.30—7.40) was observed when the recordings were started. According to available data (YANNET 1940) the normal acid base balance of the blood of the cat is characterized by a CO_2 tension of 36 mm Hg at a pH of 7.35. To restore the normal conditions according to YANNET it was necessary to inject $NaHCO_3$ into the blood stream

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Table I Systemic blood pressure responses to i v injections of noradrenaline and mean arterial blood pH during ventilation with O_2 (control period) 10 and 20% CO_2 in O_2 in cats anesthetized with sodium pentobarbital The pressor responses are given in relative units ± standard error of mean Control response during ventilation with O_2 = 100

% CO in insp gas	Number of animals	Arterial blood pH	Dose of noradr µg/kg	Systemic blood pressure response
0	11	7 38	0 17—2 0	100
10	5	7 04		55 ± 5 8
20	10	6 84		27 ± 3 6

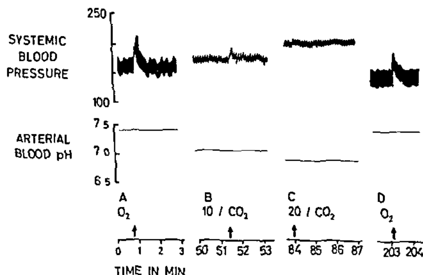


Fig 6 Cat 3 4 kg Systemic blood pressure responses to i v injections of noradrenaline during ventilation with O (control period) 10 and 20 CO in O Each arrow indicates an injection of 1µg/kg noradrenaline

increased 3 7 times range 2 4—4 5 3 cats) during ventilation with 10% CO and 37 times range 15—50 3 cats) during ventilation with 20% CO The same result was obtained when i v infusions of noradrenaline were used instead of injections

Peripheral vascular beds

The reactivity of the following vascular beds was studied before and during respiratory acidosis a) skinned hind leg (7 cats) b) unskinned hind leg 10 cats) c) kidney (3 cats)

was returned to the animal either via the proximal stump of the same vein or by another vein

The upper part of the femoral artery was ligated and then two cannulae were inserted, one on either side of this ligature The cannulae were connected by a polyethylene tube and from a T tube in the small loop thus formed, the arterial pressure was recorded When flow recordings were to be made in both hind legs at the same time a cannula was inserted into the other femoral artery and this was also connected into the loop By partial clamping of the upper part of the loop the inflow pressure to both legs could then be changed to the same degree

In some experiments the arterial blood flow was kept constant with the help of a Sigmamotor pump In the cases the arterial loop was arranged in the same way as described in chapter II

2 Results

Systemic circulation

The induction of respiratory acidosis was usually followed by an increase in systemic blood pressure When the operative procedure was extended to include denervation of the suprarenal glands and resection of a part of the left lumbar sympathetic chain a decrease in the systemic blood pressure was obtained in 9 out of 19 cats In the reserpine treated animals the systemic blood pressure always decreased during respiratory acidosis

The heart rate usually decreased during respiratory acidosis In cats treated with reserpine respiratory acidosis always induced a decrease in heart rate The decrease was so marked in these animals that the induction of the acidosis had to be made gradually since otherwise a temporary cardiac arrest would occur Cardiac arrythmias were only occasionally seen and then in the form of single extrasystoles

The end tidal CO_2 tension was measured in 9 cats and increased from 35 mm Hg (range 33—39) during the control period to 85 (range 71—96 6 cats) and 166 mm Hg (range 154—178, 9 cats) during ventilation with 10 and 20% CO_2 in O_2

The mean values of arterial blood pH recorded in the different groups of cats are given in the respective figures

The systemic blood pressure response to i v injections of noradrenaline was followed during periods of acidosis in 11 cats Fig 6 shows a representative experiment and the results are summarized in Table I To keep the blood pressure responses unchanged, the dose of noradrenaline had to be

Table I Systemic blood pressure responses to i v injections of noradrenaline and mean arterial blood pH during ventilation with O_2 (control period) 10 and 20% CO_2 in O_2 in cats anesthetized with sodium pentobarbital The pressor responses are given in relative units ± standard error of mean Control response during ventilation with O_2 = 100

% CO in insp gas	Number of animals	Arterial blood pH	Dose of noradr µg/kg	Systemic blood pressure response
0	11	7 38	0 17—2 0	100
10	5	7 04		55 ± 5 8
20	10	6 84		27 ± 3 6

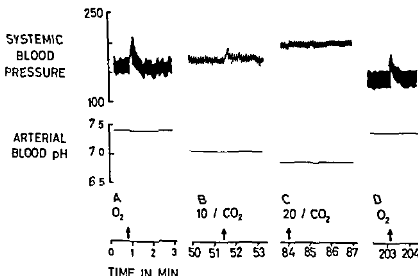


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increased 3 7 times (range 2 4—4 5 3 cats) during ventilation with 10% CO and 37 times (range 15—50 3 cats) during ventilation with 20% CO The same result was obtained when i v infusions of noradrenaline were used instead of injections

Peripheral vascular beds

The reactivity of the following vascular beds was studied before and during respiratory acidosis a) skinned hind leg (7 cats) b) unskinned hind leg 10 cats) c) kidney (3 cats)

The arterial blood flow was adjusted at the start of an experiment so that the perfusion pressure equalled the systemic blood pressure The mean blood flow was 8 3 (*range* 5 9—10 4), 9 4 (*range* 6 6—14 4) and 25 (*range* 20—30 6) ml/min in the respective groups (1—3)

Ventilation with 10 and 20% CO in O caused an increase in the resistance of the perfused leg preparations resulting in an increase in the perfusion pressure This pressure increase amounted to 35 (5—90) and 80 (45—125) mm Hg in the skinned leg and to 35 (0—50) and 60 (—25—130) mm Hg in the unskinned leg During ventilation with 10% and 20% CO the perfusion pressure decreased by 14 (5—28) and 13 mm Hg in the kidney

Before and during the acidotic period i a injections of noradrenaline were given repeatedly and the effect on the perfusion pressure was observed The dose of noradrenaline used for the i a injections was adjusted to give about

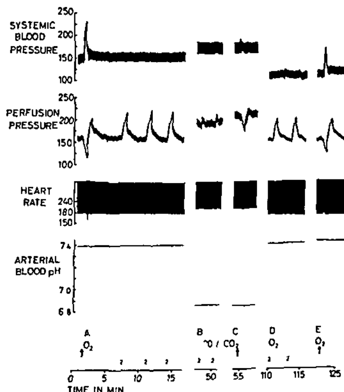


Fig 7 Cat 2 5 kg The left hind leg perfused with the cats own blood at a constant flow of 7 2 ml/min
Systemic and perfusion pressure responses to i v and i a (to the perfused leg) injections of noradrenaline during ventilation with O_2 (control period) and 20% CO in O
1 i v injection of 0 5 μg/kg noradrenaline
2 i a injection of 0 025 μg/kg noradrenaline

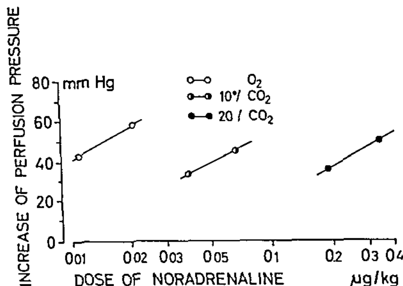


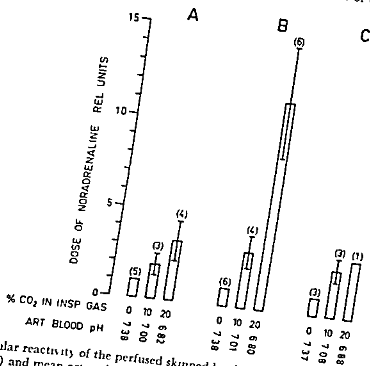
Fig 8 Cat 2 2 kg Dose response curves obtained with i a injections of noradrenaline in the perfused hind leg during ventilation with O (control period) 10 and 20% CO in O The norad enaline doses were increased during the periods of ac dosis to keep the re sponses relatively unchanged

a 50 mm Hg increase in the perfusion pressure during the control period (ventilation with oxygen) In the skinned leg the dose varied from 0 036 to 0 21 µg/kg b w (mean 0 088) in the unskinned it varied from 0 008 to 0 065 µg/kg b w (mean 0 028) and in the kidney experiments the range was from 0 093 to 0 33 µg/kg b w (mean 0 20)

Along with an increasing degree of acidosis the peripheral vasoconstrictor effect of noradrenaline diminished It could sometimes be noticed however that there was a lag time up to 10 minutes between the rapid decrease in arterial blood pH and the change in reactivity The degree of the reduction was measured either as the percentual change of the response (Fig 7) or as the ratio between the doses which induced the same pressor response of 50 mm Hg (Fig 8) The results are summarized in Fig 9 and 10

From figures 9 and 10 it can be seen that even if the change in vascular reactivity was qualitatively similar quantitative differences existed between the different vascular beds The difference observed between the results obtained in the two leg preparations was significant $p < 0\ 005$ (decrease in re sponse) resp $< 0\ 05$ (equieffective doses) at ventilation with 20% CO The change in arterial pH between the two groups was not significantly different

Fig 9 Perfusion pressure responses to i a injections of noradrenaline in the perfused skinned hind leg (A) in the unskinned hind leg (B) and in the kidney (C) and mean arterial blood pH during ventilation with O_2 (control period) 10 and 20% CO_2 in O_2 The bars indicate the pressure responses in relative units Control response = 100 In this and in the following figures numerals within parenthesis indicate number of cats Standard error of means are also given



10 Vascular reactivity of the perfused skinned hind leg (A) unskinned hind leg (B) kidney (C) and mean arterial blood pH during ventilation with O (control period) nd 20% CO in O The bars indicate in relative units the dose of noradrenal[illegible] had to be injected i a to give a 50 mm Hg increase of the [illegible]
=1

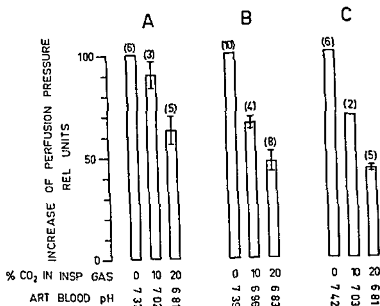


Fig. 11. Perfusion pressure responses to i.a. injections of noradrenaline in the sympathetically denervated skinned (A) and unskinned (B) hind leg preparations and mean arterial blood pH during ventilation with O (control period), 10 and 20% CO in O. *For comparison the values obtained in the intact hind leg of reserpine treated animals* have been included (C). The bars indicate in relative units the pressure responses. Control response = 100.

Role of the sympathetic nerves in the diminished peripheral effect of noradrenaline

The peripheral vasoconstrictor effect of noradrenaline was studied both in the sympathetically denervated skinned (6 cats) and sympathetically denervated unskinned (13 cats) hind leg preparations during respiratory acidosis. The results obtained in the denervated animals were extended by some experiments on reserpine treated animals (6 cats) in which the unskinned hind leg was used for the perfusion.

The noradrenaline dose found to elicit a 50 mm Hg increase in the perfusion pressure on i.a. administration in the usual way were on an average 0.035 (range 0.003—0.081) and 0.033 (range 0.008—0.075) µg/kg b.w. in the skinned and unskinned hind leg preparations. After treatment with reserpine the mean dose used was 0.016 (range 0.007—0.031) µg/kg b.w. The mean arterial blood flow was for the same groups 9.8 (range 5.9—14.4), 13.0 (range 8.7—20.0) and 12.6 (range 6.0—14) ml/min respectively.

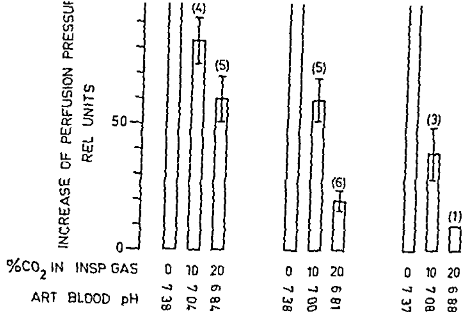


Fig 9 Perfusion pressure responses to i a injections of noradrenaline in the perfused skinned hind leg (A) in the unskinned hind leg (B) and in the kidney (C) and mean arterial blood pH during ventilation with O_2 (control period) 10 and 20% CO in O_2 The bars indicate the pressure responses in relative units Control response = 100 In this and in the following figures numerals within parenthesis indicate number of cats Standard error of means are also given

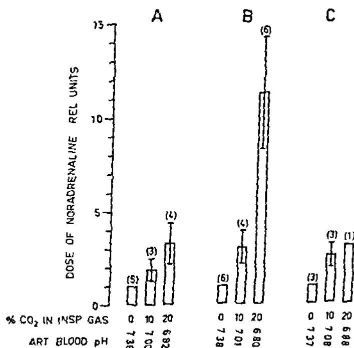


Fig 10 Vascular reactivity of the perfused skinned hind leg (A) unskinned hind leg (B) *and kidney (C) and mean arterial* blood pH during ventilation with O (control period) 10 and 20% CO in O The bars indicate in relative units the dose of noradrenaline which had to be injected i a to give a 50 mm Hg increase of the perfusion pressure Control dose = 1

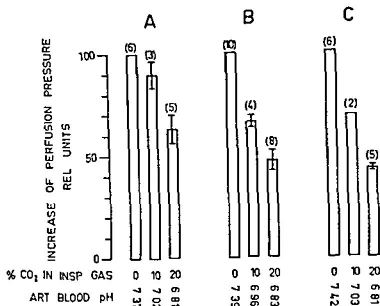


Fig 11 Perfusion pressure responses to i a injections of noradrenaline in the sympathetically denervated skinned (A) and unskinned (B) hind leg preparations and mean arterial blood pH during ventilation with O (control period) 10 and 20% CO in O For comparison the values obtained in the intact hind leg of reserpine treated animals have been included (C) The bars indicate in relative units the pressure responses Control response = 100

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The noradrenaline dose found to elicit a 50 mm Hg increase in the perfusion pressure on i a administration in the usual way were on an average 0 035 (range 0 003—0 081) and 0 033 (range 0 008—0 075) µg/kg b w in the skinned and unskinned hind leg preparations After treatment with reserpine the mean dose used was 0 016 (range 0 007—0 031) µg/kg b w The mean arterial blood flow was for the same groups 9 8 (range 5 9—14 4) 13 0 (range 8 7—20 0) and 12 6 (range 6 0—14) ml/min respectively

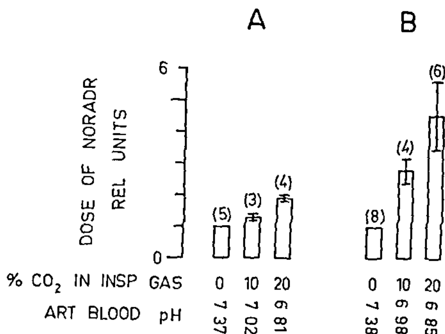


Fig 12 Vascular reactivity of the perfused sympathetically denervated skinned (A) and unskinned (B) hind leg preparations and mean arterial blood pH during ventilation with O (control period) 10 and 20% CO_2 in O_2 The bars indicate in relative units the dose of noradrenaline which had to be given i a to give a 50 mm Hg increase of the perfusion pressure Control dose = 1

In the denervated hind leg preparations both vasoconstriction and dilatation were seen during respiratory acidosis The usual finding was a decrease in resistance Simultaneously the overall effect of noradrenaline decreased as can be seen in Fig 11 and Fig 12

When the results obtained in the denervated skinned and unskinned leg preparations are compared the difference is less marked than the difference between the same preparations with intact sympathetic supply It is significant when the ratios between equieffective doses during ventilation with 20% CO (see Fig 12) are used for comparison ($p = 0\ 005$) The perfusion pressure responses to noradrenaline are significantly less impaired in the sympathetically denervated hind leg than in the innervated preparation during ventilation with 20% CO ($p < 0\ 005$ and $< 0\ 05$) A similar difference is also apparent between the results obtained in the innervated and denervated skinned hind leg preparations

Distribution of blood flow

As distinct from the other vascular beds perfused the unskinned hind leg preparation consists of two main different types of vessel the skin vessels and

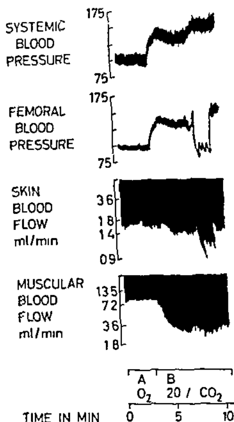


Fig 13 Cat 3 0 kg Venous outflow from the skinned hind leg (mus le blood flow) and from the paw (skin blood flow) dur ng ventilation with O (A) and 20% CO in O (B) During the ac dotic period the arterial inflow to the legs is partly occluded during 1½ min

the muscle vessels It seemed conceivable that the induced acidosis might cause an uneven vasoconstriction in these two vascular beds leading to an altered distribution of the arterial blood stream and the injected noradrena line This could probably interfere with the overall effect of noradrenaline on the perfused leg Before the results were interpreted it was therefore necessary to investigate whether such a changed distribution of blood really took place

In the first experiments carried out the venous outflow from skin and muscle vascular beds were measured simultaneously It was then observed that during respiratory acidosis the blood flow through the muscle vascular bed was markedly reduced in spite of the increased systemic blood pressure

On the other hand the skin blood flow either remained unchanged or increased (Fig 13) The small vasoconstriction in the skin vascular bed in Fig 13 is not revealed until the arterial loop is partially clamped reducing the blood pressure to the same value as before the induction of the acidosis

In experiments employing the constant flow perfusion technique this uneven degree of vasoconstriction led to a changed distribution of the blood stream from the muscle vessels to the skin In the intact hind leg the venous outflow from the paw thus increased 100—130% during ventilation with 20% CO After section of the sympathetic nerves to the perfused leg this type of changed distribution of the blood flow did not occur

3 Discussion

Respiratory acidosis is known to exert many different actions on the circulatory system The overall effect, as reflected by the systemic blood pressure, represents a balance between three components First, a direct peripheral vasodilatating effect (BAYLISS 1901, FLEISCH 1921, FLEISCH et al 1932, McDOWALL *1930*, HERMANN, MORIN and VIAL *1935*, SCHNEIDER and WILDBOLZ 1937, STECK and GELLHORN 1939) Second, a vasoconstrictor effect initiated from the vasomotor centre (HEYMANS, NOWAK and SAMAAN 1934, McDOWALL 1935, GELLHORN and LAMBERT 1939) and third, to a lesser extent, a vasoconstrictor effect exerted via the chemoreceptors (HEYMANS et al 1932, HEYMANS and BOUCKAERT 1933, BERNTHAL 1938) The usual result reported of the effect of respiratory acidosis is an increase in systemic blood pressure, *this is also the finding of the present investigation It is* known, however, that an increasing depth of narcosis can diminish or even reverse the blood pressure reaction brought about by an excess of CO due to depression of the vasomotor center allowing the peripheral vasodilator action to predominate (CATHCART and CLARK 1914, GELLHORN and LAMBERT 1939) It is possible that the decrease in the number of animals reacting with an increase in systemic blood pressure during respiratory acidosis *in the sympathetically denervated cats can be attributed to a deeper* narcosis than in the un denervated group It might also be attributed to the denervation of the adrenal glands

In cats pretreated with reserpine a decrease in systemic blood pressure was obtained during acidosis This is in accordance with earlier observations for example on cats treated with ergotamine (e g EULER and LILJESTRAND 1946) or on dogs subjected to total sympathectomy (PAGE and OLMSTED *1951*)

The pronounced bradycardia observed in reserpine treated animals at the induction of respiratory acidosis indicates that sympathetic nerves may play some role in the performance of the heart during acidosis This confirms earlier results obtained in the denervated heart lung preparation of dogs (NAHAS and CAVERT 1957) It is interesting to observe, however that if the respiratory acidosis is gradually induced the reserpine treated cats survived exposure to 20% CO

The decreased systemic blood pressure responses to i v noradrenaline injections during respiratory acidosis confirms the findings of previous workers (for references see chapter I) However these former studies have not been quantitative and so although the results are similar qualitatively it is not possible to say whether or not there is any quantitative difference between their work and the present work

The reactivity of the perfused vascular beds to noradrenaline decreased during acidosis and the decrease was related to the degree of acidosis It was however smaller than and can probably only partly explain the decreased systemic blood pressure responses observed along with periods of acidosis

Two recent investigations NASH and HEATH (1961) and BYGDEMAN and EULER (1962) reported a diminished effect of noradrenaline in the vascular beds perfused by the carotid artery of the dog and the femoral artery of the cat during acidosis At variance with the results obtained in the present study FLEISHMAN et al (1957) using the dog foreleg and BOHR and MC VAUGH (1959) and BOHR (1962) using the rat and rabbit hind legs observed an unchanged or even increased degree of vasoconstriction following i a injections of noradrenaline or adrenaline during periods of respiratory acidosis This difference might in the case of FLEISHMAN et al be explained by the short time of exposure to 20% CO_2 (7½ minutes) they used Even if this time was enough to allow the main change in arterial blood pH to occur it is probable that it is too short to alter the vascular reactivity As regards the studies of BOHR and MCVAUGH and BOHR no information is given about the time of exposure to the different changes in pH However in their experiments modified Krebs solution was used for the perfusion and it is possible that the relative degree of tissue hypoxia associated with this perfusion technique may have interfered with the vascular reactivity Of course it is also conceivable that the discrepancy may be explained by species differences and until further information is available a discussion of this problem can be only speculative

The change in vascular reactivity differed quantitatively in the different vascular beds and it is interesting to note that the muscle vessels which are normally exposed to a local decrease in pH during muscular work preserved

On the other hand the skin blood flow either remained unchanged or increased (Fig 13) The small vasoconstriction in the skin vascular bed in Fig 13 is not revealed until the arterial loop is partially clamped reducing the blood pressure to the same value as before the induction of the acidosis

In experiments employing the constant flow perfusion technique this uneven degree of vasoconstriction led to a changed distribution of the blood stream from the muscle vessels to the skin In the intact hind leg the venous outflow from the paw thus increased 100—130% during ventilation with 20% CO After section of the sympathetic nerves to the perfused leg this type of changed distribution of the blood flow did not occur

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The difference between the results obtained in the innervated and denervated skinned hind leg preparations cannot be explained by a changed distribution of the blood flow In chapter III it was shown however that an increased tone in a perfused muscle vascular bed could decrease the effect of i.a injections of noradrenaline It is probable that the difference between the innervated and denervated skinned hind leg preparations mentioned can at least partly be explained by the nervous vasoconstriction elicited by the acidosis only in the innervated preparation To what extent this mechanism might explain the difference between the sympathetically innervated and denervated whole leg preparations has not been defined but it is thought that it will play some role

their reactivity better than the other vascular beds studied in this investigation

There was a significant difference between the decrease in reactivity observed in the skinned hind leg *and the decrease found in the unskinned hind* leg preparation during acidosis This at first seemed difficult to explain even although one assumed that during respiratory acidosis the reactivity of the skin vessels was more diminished than the reactivity of the muscle vessels In two parallel coupled vascular beds as in the hind leg, the reactivity of the muscle vascular bed, through which most of the blood flows, will largely determine the vascular reactivity of the whole leg What happens to the reactivity of the skin vessels will be less important The changed distribution of the blood flow, observed in the intact leg preparation during acidosis, will *naturally change this picture so that a marked decrease in the reactivity of* the skin vessels will now influence the vascular reactivity of the whole leg more than would be expected with the original distribution of the blood flow between skin and muscle vessels

A concomitant constriction of muscle vessels and dilatation of skin vessels during periods of respiratory acidosis has been reported earlier in dogs and cats (BETZ 1962), in rats (TAKACS and KALLAY 1963) and in humans (McARDLE and RODDIE 1958) The same pattern can also be obtained in nonanesthetized animals (BETZ 1962) and humans (McARDLE et al 1957) *It has further been shown by FOLKOW, JOHANSSON and LÖFVING (1961)* that during reflex activation of the sympathetic nervous system, vasoconstrictor nerves to different vascular beds can be activated independently

It will perhaps be claimed that the changed distribution of the blood obtained in the perfusion experiments on the unskinned leg is an artifact due to the constant blood flow Also, Fig 13 does not show an increased blood flow through the skin vessels However, even in this case, the relationship between the skin and muscle blood flow changed from 1/5 to 1/2 during *acidosis This means that in this case also the importance of the changes in the resistance of the skin vessels, in accounting for the total vascular resistance* of the leg, had increased considerably, even if the absolute volume of blood flow had not increased

The decrease in vascular reactivity to noradrenaline observed during acidosis in the sympathetically denervated skinned and unskinned hind legs was less than in the same preparations with an intact nervous supply The results obtained in the denervated skinned leg and in the denervated unskinned leg preparations were *still significantly different in spite* of the fact that the requirements for a changed distribution of the blood flow did not exist This gives further support for the assumption that the reactivity of skin vessels is more decreased than that of muscle vessels during acidosis

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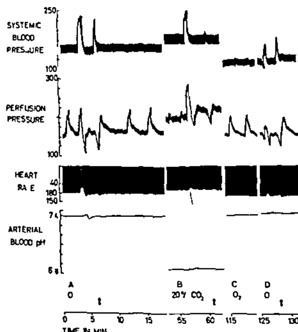


Fig 14 Cat 2 5 kg Atropine 0 2 mg/kg i v Left hind leg perfused at a constant blood flow of 7 2 ml/min. Systemic and perfusion pressure responses to i v and i a (to the perfused leg) injections of noradrenaline and bilateral occlusion of the common carotid arteries during ventilation with O (control period) and 20 % CO in O
1 i a injection of 0 0?5 μg/kg noradrenaline ? carotid occlusion test 3 i v injection of 0 5 μg/kg noradrenal ne

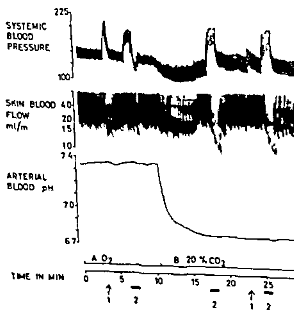


Fig 15 Cat 2 0 kg Atropine 0 ? mg/kg i v
Skin (paw) blood flow and systemic blood pressure responses to i v injections of noradrenaline (1 μg/kg at 1) and bilateral occlusion of the common carotid arteries (2) during ventilation with O (control period) and ?0 CO in O

CHAPTER V

THE EFFECT OF OTHER VASOCONSTRICTOR STIMULI DURING RESPIRATORY ACIDOSIS

The vasoconstrictor effect of carotid occlusion and stimulation of the sympathetic chain were studied in the perfused hind leg before and during exposure to respiratory acidosis, and they were compared to the effect of noradrenaline. The influence of respiratory acidosis on the vasoconstrictor effect of angiotensin was also evaluated in the unskinned hind leg.

In the experiments where the skin blood flow (from the paw) was measured this was made with a drop recorder on the venous side in the same way as described in chapter IV.

I Results

Carotid occlusion

The systemic blood pressure response to bilateral occlusion of the common carotid arteries was found to remain almost unchanged during periods of respiratory acidosis (ventilation with 20% CO_2 in O_2). On the other hand the pressor effect of i.v. noradrenaline injections decreased in the same experiments in the usual way (Fig. 14). In the perfused hind leg during acidosis occlusion of the common carotid arteries caused a similar response as during the control period, while, at the same time, the effect of noradrenaline was diminished as reported in the preceding chapter (Fig. 14).

The venous outflow from the paw of the hind leg was measured in another series of experiments (6 cats), together with the systemic blood pressure and arterial blood pH (Fig. 15). The systemic blood pressure response to occlusion of the carotid arteries and i.v. injections of noradrenaline changed during acidosis as described in the preceding paragraph. The skin blood flow however, which remained unchanged during occlusion of the carotid arteries at a normal pH, decreased when the same test was repeated during acidosis (Fig. 15). This decrease in blood flow could not always be obtained but was clearly seen in 3 out of 6 experiments.

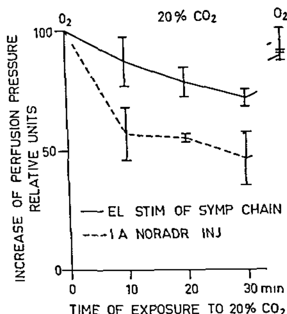


Fig 17 Perfusion pressure responses to i a injections of noradrenaline and electrical stimulation of the ipsilateral sympathetic chain in the perfused unskinned hind leg during ventilation with O_2 (control periods) and 20% CO_2 in O_2. The pressure responses are given in relative units control response = 100. Each point represents a mean value from results obtained in 4—6 cats. Standard error of means are also given

In Fig 17 the perfusion pressure responses induced by i a injections of noradrenaline and sympathetic chain stimulation are plotted against time of exposure to 20% CO_2. The dose of noradrenaline and the frequency of stimulation were chosen so that in each cat the effect on the perfusion pressure was almost equal during the control period. The different change in reactivity was significant at the 5% level ($p < 0.05$). To obtain the same increase in the perfusion pressure during ventilation with 20% CO_2 in O_2 as during the control period the noradrenaline doses had to be increased 3.7 times (range 2.0—6.2) and the stimulation frequences 1.5 times (range 1.3—1.8) ($p < 0.02$)

Angiotensin

The vasoconstrictor effect of angiotensin was tested before and during respiratory acidosis using the sympathetically innervated (4 cats) and denervated hind leg preparations (4 cats). The dose of angiotensin was in the

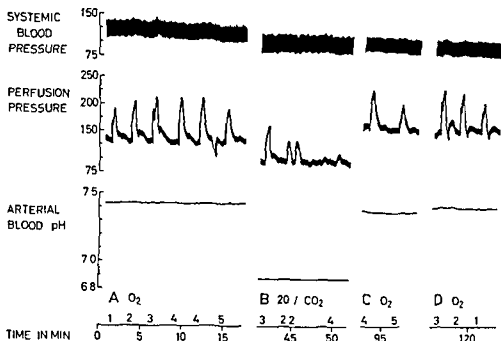


Fig 16 Cat 3 6 kg Atropine 0 2 mg/kg i v Left hind leg perfused with a constant blood flow of 12 3 ml/min
Perfusion pressure responses to electrical stimulation of the left sympathetic chain at the midlumbar level and i a noradrenaline injections during ventilation with O_2 (control periods A C and D) and 20% CO in O_2 (B)
1 el stimulation 5 V 0 2 imp/sec duration 30 sec
2 el stimulation 5 V, 0 4 imp/sec duration 30 sec
3 el stimulation 5 V 0 8 imp/sec duration 30 sec
4 i a injection of noradrenaline 0 007 μg/kg
5 i a injection of noradrenaline 0 004 μg/kg

Electrical stimulation of the sympathetic vasoconstrictor nerves

Electrical stimulation of the sympathetic chain at the midlumbar level was employed in 6 cats to obtain a more defined stimulation, than reflex activation of the vasoconstrictor nerves leading to the ipsilateral hind leg The perfusion pressure responses were compared with those of i a injections of noradrenaline before and during respiratory acidosis Fig 16 shows examples from an experiment of this kind An i a injection of noradrenaline of 0 007 μg/kg b w (at 4, section A) gave, in this experiment, a perfusion pressure response, which equalled stimulation of the sympathetic chain with a frequency of 0 8 imp/sec (at 3 section A) during ventilation with oxygen During ventilation with 20% CO the effect of i a injections of noradrenaline was almost abolished while the pressor response induced by electrical stimulation decreased less

and pulse pressures observed during acidosis Both these changes will tend to increase the systemic and perfusion pressure responses to occlusion of the common carotid arteries (HEYMANS and NEIL 1958) It is difficult to believe however, that this is enough to compensate the decreased reactivity of the circulatory system found during ventilation with 20% CO (chapter IV) when tested with i v injections of noradrenaline

LOFVING (1961) reported that bilateral occlusion of the common carotid arteries induces a greater constriction of the muscle vessels than of the skin and intestinal vessels and leaves the tonus of the renal vessels unchanged FOLKOW JOHANSSON and LOFVING (1961) suggested that this discrepancy could be explained by different thresholds to excitatory stimuli between the different medullary neuron pools controlling these functionally different vascular beds They showed that other excitatory impulses can so affect the excitability of inactive neuron pools that they may be activated by otherwise subthreshold stimuli A decrease in ventilation thus changed the effect of a carotid occlusion on the renal vessels from that of no change in tonus to a marked vasoconstriction The same change in reactory pattern could be observed in the present study in a skin vascular bed during respiratory acidosis This indicated that an accumulation of CO even without a concomitant hypoxia might constitute an excitatory stimulus of the kind mentioned above

In the preceding paragraphs some explanations have been discussed for the unchanged pressor responses to bilateral occlusion of the common carotid arteries during respiratory acidosis These explanations will all result in an increased vasoconstrictor discharge during carotid occlusion at a low pH in comparison with that induced at a normal pH possibly compensating the decreased cardiovascular reactivity

Another way to explain the unchanged pressor responses to carotid occlusion during acidosis is to assume that the effect of injected noradrenaline *is influenced* by respiratory acidosis in a way different from the locally released transmitter The experiments in which electrical stimulation of the *sympathetic* chain were performed suggested that such a difference can be recognized To my knowledge no earlier study has been made on the effct of vasoconstrictor nerve stimulation during respiratory acidosis BURGET and CRISLER (1927) have observed however that the systemic blood pressure response to electrical stimulation of the sympathetic cord in the neck diminished during periods of decreased *ventilation*

It is difficult to believe that the difference in result between nerve stimulation and i a injections of noradrenaline can be due to a changed excitability of the nerves since a supramaximal voltage strength has been used But if there is a change in the excitability of the nerves then one would ex

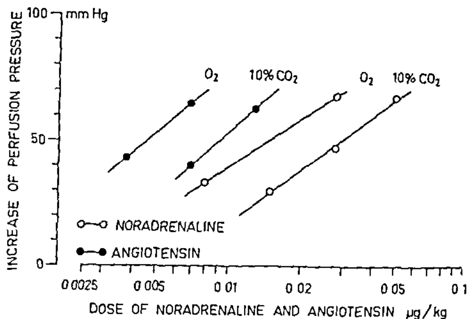


Fig 18 Cat 3 0 kg Dose response curves obtained with i a injections of angiotensin and noradrenaline in the perfused sympathetically denervated unskinned hind leg during ventilation with O_2 (control period) and 10% CO in O Note the equal horizontal distance between corresponding angiotensin and noradrenaline curves

innervated group 0 005 µg/kg (range 0 003—0 008) and in the denervated group 0 017 µg/kg (range 0 004—0 038) The perfusion pressure responses decreased during induced respiratory acidosis (ventilation with 10 and 20% CO in O) to a similar extent as had earlier been shown for noradrenaline To keep the response unchanged during acidosis the dose of angiotensin had to be increased to a similar degree as noradrenaline (Fig 18)

2 Discussion

The striking difference between the well maintained blood pressure responses to bilateral occlusion of the common carotid arteries and the decreased effect of injected noradrenaline during acidosis is somewhat surprising in view of the general assumption that the effect of carotid occlusion on the cardiovascular system is mediated by the adrenergic neurotransmitter DUNER and EULER (1959) observed the same difference when studying the systemic blood pressure responses of i v noradrenaline injections during periods of reduced ventilation in the cat

This difference may partly be explained by the increased systemic blood

CHAPTER VI

THE VASOCONSTRICTOR EFFECT OF NORADRENALINE DURING RESPIRATORY ALKALOSIS AND NON RESPIRATORY CHANGES IN THE ACID BASE BALANCE OF THE BLOOD

The vasoconstrictor effect of noradrenaline was recorded in the perfused skinned hind leg of the cat a) during respiratory alkalosis b) during non respiratory alkalosis induced by infusion of 0 6 M $NaHCO_3$ c) during shifts in the CO_2 tension of the blood In the latter case the pH of the blood was kept constant by infusion of 0 2 M HCl

The ipsilateral sympathetic chain was cut so that variations in the systemic blood pressure concomitant with the acid base shifts would not cause reflex changes in the peripheral resistance of the perfused vascular bed

1 Results

The mean arterial blood flow to the perfused leg was 8 3 ml/min (range 4 7—10 4) The dose of noradrenaline which had to be injected i a to increase the perfusion pressure 50 (40—60) mm Hg had a mean of 0 079 (range 0 015—0 38)

Respiratory alkalosis

The effect of respiratory alkalosis was investigated in 10 cats These were divided into two groups with 5 cats in each The degree of hyperventilation was adjusted to give a mean arterial blood pH of 7 56 (range 7 52—7 63) in the first group and a mean arterial blood pH of 7 80 (range 7 71—8 00) in the second group The mean end tidal CO tension decreased from 40 mm Hg (38—41) to 19 (17—20) and 13 mm Hg (11—16) The rate and volume of respiration were kept constant during both the control period and the period of alkalosis During the control period the pH and CO_2 values were kept normal by the addition of 3—5% CO_2 to the inhaled oxygen

Respiratory alkalosis usually caused a decrease in both the systemic and perfusion blood pressures The perfusion pressure decrease was in mean 8

pect a decrease in the nerve excitability, since it has been shown that respiratory acidosis decreases the excitability of peripheral nerves (LORENTE DE NO 1947, MONNIER 1952, CORABOEUF 1951, 1954)

Decreased systemic blood pressure responses to angiotensin during acidosis has earlier been observed by PAGE and OLMSTED (1951) and MIESMANN et al (1961) The present experiments show that this decreased effect could partly be explained by a decrease in peripheral vascular reactivity to about the same extent as has been shown for noradrenaline

HALPERN (1956 a and b), HALPERN, MAYER and BUCNARD (1956) and HALPERN et al (1959) reported that "respiratory acidosis" decreased the contractions induced by histamine in the isolated guinea pig ileum and by acetylcholine in the uterus from rat, rabbit and guinea pig The effect of other stimulating substances tested was not affected, however These results indicate that the effect of the acidosis is specific and only influences the sensitivity of cellular receptors for certain substances and not the ability of the smooth muscle itself to contract As far as the vascular smooth muscle is concerned the results obtained in this present investigation do not give evidence for a decreased sensitivity of only one type of specific receptor However, if it is accepted that respiratory acidosis will influence the action of circulating noradrenaline more than the locally released neurotransmitter, the factor causing this difference might influence the effect of other injected vasoconstrictor substances as well

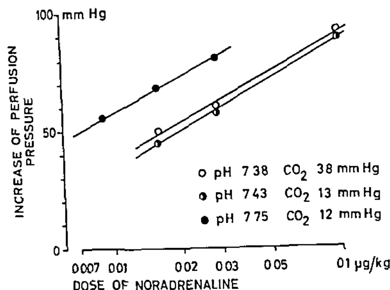


Fig 21 Cat 4 3 kg Dose response curves obta ned with i a injections of noradrenaline in the perfu ed skinned hind leg Sympathetic cha n cut O—O normal acid base balance ◐—◐ compensated non respiratory acidosis ●—● uncompensated respiratory alkalosis

mm Hg (range —55— + 20) in the first group and 13 mm Hg (range —40 — + 10) in the second group The perfusion pressure response to the i a injection of noradrenaline was increased (Fig 19) The results are summarized in Fig 20

Compensated non respiratory acidosis

In seven of the cats used in the experiments described in the preceding paragraph a corresponding decrease in CO tension was also induced while the arterial blood pH was kept constant In four cats the effect of noradrenaline decreased or was unchanged while an increased effect was observed in three Only in one of these three cats was the increase similar to that obtained during a subsequent alkalosis Dose response curves from one experiment are shown in Fig 21 In this case no change in reactivity was observed during compensated non respiratory acidosis Respiratory alkalosis however shifted the dose response curve to the left as described above

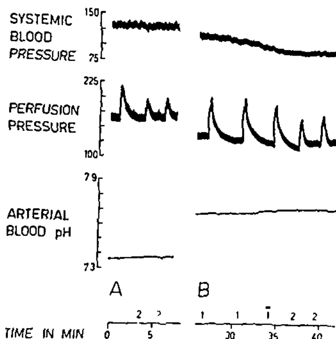


Fig 19 Cat 4 1 kg The skinned sympathetically denervated hind leg perfused with a constant blood flow of 8 7 ml/min Effect of respiratory alkalosis on the perfusion pressure responses to i a injections of noradrenaline A P_{CO} in end tidal air 41 1 mm Hg B P_{CO} in end tidal air 15 9 mm Hg
1 Noradraline inj ction i a 0 02 μg/kg 2 Noradrenaline injection i a 0 006 μg/kg

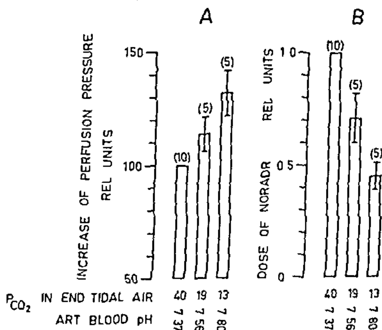


Fig 20 Effect of respiratory alkalosis on mean arterial blood pH mean end tidal CO tension and on the vascular reactivity of the perfused sympathetically denervated skinned hind leg A The bars indicate in relative units the perfusion pressure responses to nor adrenaline Control response = 100 B The bars indicate in relative units the dose of noradrenaline which had to be injected i a to give a perfusion pressure response of 50

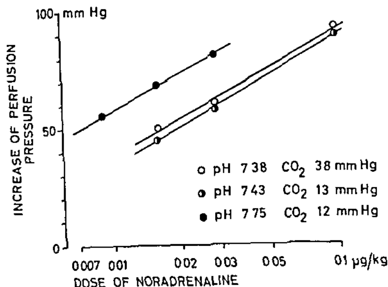


Fig 21 Cat 4 3 kg Dose response curves obtained with i a injections of noradrenaline in the perfused skinned hind leg Sympathetic chain cut o—o normal acid base balance o—o compensated non respiratory acidosis •—• uncompensated resp ratory alkalosis

mm Hg (range —55—+20) in the first group and 13 mm Hg (range —40 —+10) in the second group The perfusion pressure response to the i a injection of noradrenaline was increased (Fig 19) The results are summarized in Fig 20

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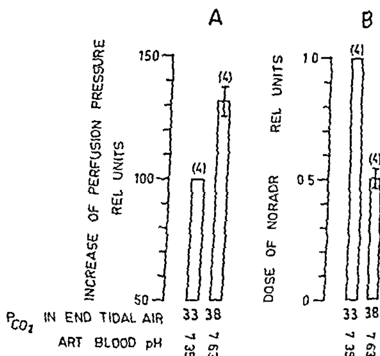


Fig 22 Effect of non respiratory alkalosis on mean arterial blood pH mean end tidal CO_2 tension and on the vascular reactivity of the perfused sympathetically denervated skinned hind leg
A The bars indicate in relative units the perfusion pressure responses to noradrenaline Control response = 100
B The bars indicate in relative units the dose of noradrenaline which had to be injected i a to give a perfusion pressure of 50 mm Hg Control dose = 1

Non respiratory alkalosis

During a period of non respiratory alkalosis (4 cats) the effect of noradrenaline increased (*Fig* 22) In two of the cats this was however preceded by a transient decrease in the effect of noradrenaline The mean end tidal CO tension changed from 33 mm Hg (range 32—35) to 38 mm Hg (36—39) Arterial blood pH increased from 7 35 (7 34—7 36) to 7 63 (7 58—7 65) The perfusion pressure decreased at the induction of the alkalosis The decrease was 14 mm Hg (range —35—+25) at the time when the test doses of noradrenaline were given

2 Discussion

In the present experiments a period of respiratory alkalosis regularly decreased the systemic blood pressure Previous reports of systemic blood pressure during respiratory alkalosis have been conflicting This is perhaps not

surprising, since the periods of alkalosis have been induced in different ways and their degree and duration have varied markedly (cf BROWN 1953) However the usual finding in muscle vascular beds is a vasodilatation and it does not seem to matter whether the sympathetic nerve supply is intact or not (CLARKE 1952 BURNUM, HICKMAN and McINTOSH 1954 RODDIE SHEPHERD and WHELAN 1957) This is in agreement with the results of the present study The dilatating effect of non respiratory alkalosis on muscle vessels confirms earlier results on dogs (DEAL and GREEN 1954)

Respiratory alkalosis has been reported in dogs, to increase the effect of noradrenaline on the vascular bed perfused by the common carotid artery (NASH and HEATH 1961) In their work the increased effect of noradrenaline during alkalosis was partly explained by an increased vascular reactivity On the other hand their results could also be explained by the marked decrease in flow through the vascular bed resulting in an increase in the local concentration of the injected noradrenaline

Respiratory alkalosis has also been reported to decrease the response of the blood vessels to adrenaline in the isolated perfused rat hind leg (BOHR and McVAUGH 1959) However a period of alkalosis should induce a vasoconstriction of the skin vessels and dilatate the muscle vessels—provided the results obtained in dogs (DEAL and GREEN 1954) and humans (CLARKE 1952) can be applied to rats This would make the result of a comparison between the effect of i a noradrenaline injections at a normal and at an increased blood pH difficult to interpret since the distribution pattern of injected noradrenaline between skin and muscle vessels may have changed

The increased effect of noradrenaline on the muscle vascular bed observed in the present study during alkalosis indicates that a change although small in vascular reactivity occurs The increased effect of noradrenaline could not be explained by the vasodilatation during alkalosis (cf chapter IV) since this was too small and the change in reactivity was not correlated with the degree of dilatation in different experiments

Both respiratory and non respiratory alkalosis increased the effect of noradrenaline This suggests that it is the blood pH which is mainly responsible for the changes observed in vascular reactivity during acid base shifts *in vivo* Changes in CO_2 tension may thus influence the effect of noradrenaline through its ability to change the blood pH The fact that a period of compensated non respiratory acidosis did not increase the effect of noradrenaline to the same extent as a period of respiratory alkalosis also favours this view

Further evidence can be obtained from investigations dealing with the effect of noradrenaline on the systemic blood pressure during acid base shifts

Thus MERSMANN, BRAASCH and HILPERT (1961) reported an enhanced response to noradrenaline in dogs during respiratory acidosis when 5% $NaHCO_3$ was infused NAHAS et al (1960) made the same observation using 0 33 M tris hydroxymethylaminomethane buffer and adrenaline MATTHIS (1962) observed that the amount of noradrenaline necessary to keep the blood pressure normal in patients suffering from cardiac shock along with acidosis could be decreased if the acidosis was treated with $NaHCO_3$ However, PAGE and OLMSTED (1951) found unchanged systemic blood pressure responses to noradrenaline during non respiratory acidosis in dogs while a respiratory acidosis with the same pH shift diminished the effect of noradrenaline RADER et al (1961) observed increased systemic blood pressure responses to adrenaline during non respiratory alkalosis in dogs, while during a period of respiratory alkalosis the effect remained unchanged On the other hand the same group reported decreased systemic blood pressure responses to adrenaline both during respiratory and non respiratory acidosis (HOULE et al 1957, KEITH et al 1960)

Using isolated strips from rat aorta, TOBIAN et al (1959) observed a decrease in the noradrenaline induced contractions *in vitro* both during "respiratory" and "non respiratory" types of acidosis This also speaks in favour of the causative role played by the pH

CHAPTER VII

GENERAL DISCUSSION

The results presented have shown that a shift in the acid base balance of the blood as for example during a period of respiratory acidosis markedly influences the cardiovascular effects of vasoconstrictor stimuli In the analysis special attention has been given to some vascular beds in the peripheral circulation The analysis has also included the effects of other types of alterations in the acid base balance of the blood.

During a period of respiratory acidosis the systemic blood pressure responses to i v injections and infusions of noradrenaline markedly decreased Also during the acidosis a diminished effect of injected noradrenaline was observed in some peripheral vascular beds However this peripheral decrease was not enough to explain entirely the diminished effect of noradrenaline on the systemic blood pressure during acidosis This difference may be explained by a decreased reactivity of the heart but it may also be accounted for by a decreased effect of noradrenaline on other vascular beds not investigated in the present study It is thus not possible to evaluate the relative importance of the changed reactivity of the heart and the summed change in peripheral reactivity when attempting to explain the decreased effect of noradrenaline on the circulatory system during respiratory acidosis.

The results obtained indicate that the decreased vasoconstrictor effect of injected noradrenaline during respiratory acidosis can partly be attributed to the increased activity of the sympathetic nervous system The influence of an increased sympathetic discharge on the effects of catecholamines during respiratory acidosis has been discussed in two earlier investigations.

PAGE and OLMSTED (1951) who studied the effect of vasoconstrictor substances on the blood pressure suggested that the refractory state observed during respiratory acidosis was *the result of inhibitor impulses* emanating from stimulated sympathetic ganglia This suggestion is not supported by the present findings since if this was the case a difference would have been found between the results obtained in the sympathetically denervated hind leg and in the hind leg of the animals treated with reserpine.

TENNEY (1956) on the other hand proposed from studies on the nictitat

ing membrane, that "much of the inhibition of response to administered adrenaline by CO was more apparent than real, because the CO tension itself served as a stimulus to raise the endogenous adrenaline titer and each subsequently administered test dose of adrenaline was then examined against a background of hormone action Because of the alinear shape of the adrenaline dose response curve this could be misinterpreted as inhibition However, results both from earlier work (FOLKOW and ÖBERG 1959, LAVERTY 1962, BICDEMAN *unpublished observations*) and from the present study indicate that as far as vascular reactivity is concerned the increase in tone induced by the activity in the *sympathetic vasoconstrictor nerves is more* important in explaining the decreased effect of noradrenaline than the increased background level of the transmitter

During respiratory acidosis a vasoconstriction was obtained in a muscle vascular bed while an unchanged tone was seen in a skin vascular bed This difference was probably due to a varying degree of activation, central and reflexogenic, of the different medullary neuron pools controlling these functionally different vascular beds In the hind leg, perfused at a constant rate of flow this led to a redistribution of the blood from vessels with a well maintained reactivity (muscle vessels) to vessels with a more decreased reactivity (skin vessels) This constituted the second way observed by which the sympathetic activation during respiratory acidosis could decrease the effect of injected noradrenaline It is probable that a changed distribution of the blood flow also occurs between other vascular beds during respiratory acidosis if results obtained in other species can be applied to cats It has been shown for example that some vascular beds such as the cerebral (SOKOLOFF 1960) and mesenteric (BRICKNER et al 1956) dilate, while other e g leg (LENNOX and GIBBS 1932 STICK and GELLHORN 1939) contract during acidosis If this is the case in cats it is probable that the overall effect of noradrenaline on the circulatory system will be increased or decreased depending on the reactivity of the vascular beds receiving the increased blood flow

The results obtained in the present study during acid base shifts in the blood indicate that the change in vascular reactivity, which remains after sympathetic denervation, is mainly due to the shifts in H ion concentration and not *to changes in CO tension by itself This is in agreement with results obtained in vitro* by TOBIAN, MARTIN and EILERS (1959) on aortic strips from rats, which could not be confirmed by WILLIAMSON and MOORE (1960) using strips from rabbit aorta

The decreased vascular reactivity usually observed during a period of marked compensated non respiratory acidosis also emphasizes *the importance*

of the pH of the blood in determining the response of a vascular bed to injected noradrenaline During this type of acid base shift the buffer capacity of the blood is reduced This will be followed by a decrease in the tissue and in the venous blood pH which is not apparent when arterial blood pH is measured

In the experiments where end tidal CO_2-tension was continuously recorded together with arterial blood pH a lower CO tension than that considered by previous authors to be normal was regularly noticed at the start at the recordings The CO tension was therefore adjusted to this supposedly normal value by reducing the ventilation and i v infusion of $NaHCO_3$ (chapter III) In a recent study (FINK and SCHOOLMAN 1963) it has been argued however that the normal acid base balance of cats is characterized by a CO_2-tension of 28 mm Hg (range 22—33 6) at an arterial blood pH of 7 38 (7 28—7 45) that is the same as the values observed initially in the experiments in the present study (chapter III) The difference in CO_2-tension will not, however invalidate the conclusion that the change in reactivity to injected noradrenaline which occurs in a sympathetically denervated vascular bed during acid base shifts is more closely correlated to a change in pH than to a change in CO_2 tension

Earlier studies have shown that other effects of catecholamines are also hindered during a period of respiratory acidosis The decreased effects have usually been attributed to the increased H ion concentration instead of the change in CO_2-tension DARBY et al (1960) for instance have shown that the effect of noradrenaline on ventricular isometric tension is decreased during acidosis and that the change in reactivity of the heart is correlated to the pH change It has further been reported that in dogs the increase in oxygen consumption which follows an injection of adrenaline is diminished during a period of apneic oxygenation The effect reappeared however in spite of the still increased CO_2-tension when enough tris-hydroxymethylaminomethan (THAM) was given to normalize the blood pH (NAHAS et al 1960) A similar rise in plasma adrenaline and noradrenaline was found by MORRIS and MILLAR (1962 a and b) during both respiratory and non respiratory acidosis This indicates that the activation of the sympathetic nervous system during acidosis is mainly due to the pH change and not to the CO tension by itself

On the other hand it seems as if the vascular smooth muscle in this respect behaves differently from some other types of smooth muscle In a series of studies HALPERN (1956 a and b) and HALPERN et al (1956 and 1959) showed that *in vitro* the histamine induced contraction of the guinea pig ileum and the oxytocin induced contractions of the guinea pig rabbit and rat uterus diminished during a "respiratory acidosis" This decreased contrac

tility was better correlated to the change in CO_2 tension than to the pH of the surrounding fluid

During a period of respiratory acidosis a marked and sustained vasoconstriction due to the acidosis may be observed in a perfused vascular bed At the same time the vasoconstrictor effect of injected noradrenaline may be abolished This puzzling observation could be explained by the finding that the vasoconstriction obtained in a perfused hind leg during sympathetic nerve stimulation is less impaired by a period of respiratory acidosis than the vasoconstrictor effect of injected noradrenaline Since the relationship between the response of the perfusion pressure and the vascular reactivity is probably the same, whether the reactivity is tested with injected noradrenaline or with stimulation of the vasoconstrictor nerves, the difference in these responses during acidosis must be attributed to the different sites of administration' of noradrenaline During nerve stimulation noradrenaline is liberated in close proximity to the receptor sites, while the noradrenaline in i a injections has to pass by a much longer route before it reaches the receptor Respiratory acidosis may thus in some way change the fraction of injected noradrenaline reaching the receptors or the number of receptors reached Whatever the factors involved in this mechanism one conclusion is that the ability of the vascular smooth muscle to contract *in vivo* is only slightly affected by a period of respiratory acidosis

MALTESOS and HADJIMINAS (1956) have reported that in dogs a brief electrical stimulation of the sympathetic chain at the lumbar level will strongly inhibit the vasoconstrictor effect of noradrenaline for up to 5 minutes in the ipsilateral hind leg After 5—10 minutes of sympathetic stimulation (cf chapter III) a decreased vasoconstrictor effect of noradrenaline was sometimes observed in a muscle vascular bed although the effect always reappeared in a few minutes In the experiments on the hind leg however, where i a injections of noradrenaline were alternated with short periods of electric stimulation of the ipsilateral sympathetic chain the above phenomenon was not seen It is therefore improbable that this can explain the different results obtained when the reactivity of a vascular bed during acidosis is tested with injected noradrenaline and stimulation of the vasoconstrictor nerves

LEWIS and MELLANDER (1962) have reported that during a period of decreased blood flow through a muscle vascular bed in cats the vasoconstrictor effect of sympathetic nerve stimulation was abolished From the results discussed in the above paragraphs it is likely that the decreased vasoconstrictor effect in their experiments is due not only to an acidosis but is partly due to the hypoxia

In the present study it was observed that the systemic blood pressure response to an i v infusion of noradrenaline decreased markedly during ventilation with 20% CO_2 and a more than thirtyfold increase in the dose was necessary to restore the blood pressure response If these results can be transferred to man it will be possible to draw some clinical implications

A more or less severe degree of respiratory acidosis is not uncommon during general anesthesia CO_2-tensions in the blood ranging from 120 to 200 mm Hg have been reported (DRIPPS 1947 TAYLER and ROOS 1950 BUCKLEY et al 1953 STEPHEN BOURGEOIS-GAVARDIN and DENT 1959 and SCHULTZE et al 1960) That is about the same or even above the tensions recorded in the present study In patients with acute respiratory disturbances a decrease in blood pH is also to be expected (e g MANFREDI et al 1960) It is unlikely that an infusion of noradrenaline as a means of raising a low blood pressure or improving an inadequate blood flow will be successful during these conditions unless the dose of noradrenaline is exceptionally high This will not be without harm however since it has been shown that after a period of infusion with a high dose of noradrenaline the phenomenon of post infusion hypotension will appear (DUNER and EULER 1957 and 1959) This hypotension can make it difficult to discontinue the infusion

Noradrenaline has been widely used in the treatment of different types of shock Since the term shock has been applied to hypotensive states associated with virtually every conceivable mechanism affecting blood pressure it is not surprising that the beneficial effect of a treatment with noradrenaline can vary in different cases

Favourable results of noradrenaline treatment are to be excepted in hypotensive states due to intake of poisons or drugs such as barbiturates since in these cases the failing vasomotor and cardiac nerve activity causing the hypotension can be replaced by the normal substance noradrenaline However it is also likely that a failing ventilation will be another symptom which will certainly interfere with the effect of noradrenaline

In other types of shock such as hemorrhagic oligemic and shock due to myocardial infarction the organism will probably respond to the hypotension with an activation of the sympatho-adrenal system with vasocon triction and decreased regional blood flow Under these circumstances the beneficial effect of noradrenaline treatment will be less obvious than during other hypotensive states which are due to failing vasomotor nerve activity although it is widely used In hemorrhagic and oligemic shock noradrenaline treatment may even prove harmful (NICKERSON 1962)

In these types of shock the disorder of the acid base balance to be expected is a non respiratory acidosis. Although the influence of a period of non re-

spiratory acidosis on the response of the blood pressure to injected noradrenaline has not been examined in the present study, the results obtained with other types of acid base disorder suggest that the effect of injected noradrenaline on a vascular bed changes along with changes in the blood pH An increasing degree of acidosis may therefore constitute one of the reasons for the unsatisfactory response of the blood pressure to noradrenaline treatment, which sometimes can be observed clinically during prolonged shock states of these types Although the change in response to injected noradrenaline observed in the sympathetically denervated skinned hind leg along with acid base shifts was small, it is conceivable that the longer time of exposure to the acidosis, which can be found under clinical conditions, will increase the significance of the acidosis in explaining the decreased effect of noradrenaline

In shock states due to myocardial infarction treatment of the hypotension with noradrenaline have been reported to give favourable results (SAMPSON and ZIPSER 1954) probably due to the improved blood flow through the coronary arteries In this case therefore it is important to obtain a satisfactory blood pressure response with as small a dose of noradrenaline as possible Thus it is essential to correct first, any defect of acid base balance

SUMMARY

The aim of the present investigation was to study the mechanisms by which the cardiovascular effect of vasoconstrictor stimuli are modified during respiratory acidosis and primarily the behaviour of some vascular beds in the peripheral circulation

The reactivity (responsiveness) of peripheral vessels during acid base shifts was estimated in the following vascular beds perfused *in situ* using the cats own blood and with a constant flow perfusing technique a) muscle vessels (skinned hind leg) b) skin and muscle vessels (unskinned hind leg) and c) renal vessels (kidney)

Changes in reactivity during acid base shifts were determined either as the change in the pressor response of a vasoconstrictor stimulus or as the ratio between doses elicitating the same pressor response both during the control period and during a period of changed acid base balance

Respiratory acidosis decreased the systemic blood pressure responses to injected noradrenaline and the decrease was correlated with the degree of acidosis To induce the same pressor response during ventilation with 20% CO_2 in O_2 as during a control period the dose of noradrenaline had to be increased 37 times

The decreased effect of noradrenaline on the cardiovascular system could partly be explained by a decreased reactivity of the perfused vascular beds The decrease in response to noradrenaline of the peripheral vessels was most marked in the unskinned leg less in the kidney and least in the skinned leg

The decreased reactivity of peripheral vascular beds (skinned and unskinned leg) during respiratory acidosis could partly be attributed to the activation of the sympathetic nervous system

The change in reactivity to injected noradrenaline which was observed in a sympathetically denervated vascular bed (skinned hind leg) during different types of acid base shifts of the blood was closely related to induced alterations in blood pH Changes in CO tension of the blood affected vascular reactivity mainly through its ability to change pH

The effect of injected angiotensin was decreased during acidosis to the same degree as noradrenaline

spiratory acidosis on the response of the blood pressure to injected noradrenaline has not been examined in the present study, the results obtained with other types of acid base disorder suggest that the effect of injected noradrenaline on a vascular bed changes along with changes in the blood pH An in*creasing degree of acidosis may therefore constitute one of the reasons for the* unsatisfactory response of the blood pressure to noradrenaline treatment, which sometimes can be observed clinically during prolonged shock states of these types Although the change in response to injected noradrenaline observed in the sympathetically denervated skinned hind leg along with acid base shifts was small, it is conceivable that the longer time of exposure to the acidosis, which can be found under clinical conditions, will increase the sig*nificance of the acidosis in explaining the decreased effect of noradrenaline*

In shock states due to myocardial infarction treatment of the hypotension with noradrenaline have been reported to give favourable results (SIMPSON and ZIPSER 1954) probably due to the improved blood flow through the coronary arteries In this case therefore it is important to obtain a satisfactory blood pressure response with as small a dose of noradrenaline as possible Thus it is essential to correct first, any defect of acid base balance

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I wish to express my sincere gratitude to Professor U S v Euler who has been my teacher, and under whose guidance it has been my privilege to work

I also wish to thank Mr Nils Åke Persson for his invaluable and skilful technical assistance

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The vasoconstriction caused by reflex activation or electrical stimulation of sympathetic vasoconstrictor nerves was less impaired during respiratory acidosis than the effect of injected noradrenaline This indicates that the decreased vasoconstrictor effect of injected noradrenaline during respiratory acidosis cannot be explained only by a decreased ability of the vascular smooth muscles to contract The fraction of the injected noradrenaline which reaches the receptors in an active state may also have changed

Some clinical implications of the results are briefly discussed

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